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PROTEINS ASSOCIATED WITH CELL DIFFERENTIATION

TECHNICAL FIELD

This invention relates to nucleic acid and amino acid sequences of proteins involved in cell differentiation and to the use of these sequences in the diagnosis, treatment, and prevention of cell proliferative, developmental, and neurological disorders.

BACKGROUND OF THE INVENTION

Multicellular organisms are comprised of diverse cell types that differ dramatically both in structure and function, despite the fact that each cell is like the others in its hereditary endowment. Cell differentiation is the process by which cells come to differ in their structure and physiological function. The cells of a multicellular organism all arise from mitotic divisions of a single-celled zygote. The zygote is totipotent, meaning that it has the ability to give rise to every type of cell in the adult body. During development the cellular descendants of the zygote lose their totipotency and become determined. Once its prospective fate is achieved, a cell is said to have differentiated. All descendants of this cell will be of the same type.

Human growth and development requires the spatial and temporal regulation of cell differentiation, along with cell proliferation and regulated cell death. These processes coordinately control reproduction, aging, embryogenesis, morphogenesis, organogenesis, and tissue repair and maintenance. The processes involved in cell differentiation are also relevant to disease states such as cancer, in which case the factors regulating normal cell differentiation have been altered, allowing the cancerous cells to proliferate in an anaplastic, or undifferentiated state.

The mechanisms of differentiation involve cell-specific regulation of transcription and translation, so that different genes are selectively expressed at different times in different cells. Genetic experiments using the fruit fly Drosophila melanogaster have identified regulated cascades of transcription factors which control pattern formation during development and differentiation. These include the homeotic genes, which encode transcription factors containing homeobox motifs. The products of homeotic genes determine how the insect's imaginal discs develop from masses of undifferentiated cells to specific segments containing complex organs. Many genes found to be involved in cell differentiation and development in Drosophila have homologs in mammals. For example, human homologs have recently been found for the Drosophila ash2 gene. The ash2 gene product is a transcriptional regulator of homeotic selector genes and is implicated in early development and formation of various disc patterns in the fruit fly (Ikegawa, S. (1999) Cytogenet. Cell Genet. 84:167-172). The ariadne-2 protein, a retinoic-acid inducible protein with a RING finger transcription factor motif, also has a human homolog (GenBank Entry g5453556, Homo sapiens

ariadne-2 (D. melanogaster) homolog).

There is evidence in some cases that the human genes have equivalent developmental roles as their Drosophila homologs. The human homolog of the Drosophila eyes absent gene (eya) underlies branchio-oto-renal syndrome, a developmental disorder affecting the ears and kidneys (Abdelhak, S. et al. (1997) Nat. Genet. 15:157-164). The Drosophila slit gene encodes a secreted leucine-rich repeat containing protein expressed by the midline glial cells and required for normal neural development. Two mammalian homologs, SLIT1 and SLIT 2, have recently been identified in both humans and mice. In mice both genes are expressed during CNS development in the floor plate (the vertebrate equivalent of midline glial cells), roof plate and developing motor neurons, suggesting a conservation of protein function between Drosophila and mammals (Holmes, G. P. (1998) Mech. Dev. 79:57-72).

At the cellular level, growth and development are governed by the cell's decision to enter into or exit from the cell cycle and by the cell's commitment to a terminally differentiated state. The schlafen family of genes, a novel family of at least 7 members, are involved in maintenance of T cell quiescence. These genes are differentially regulated during thymocyte maturation and are preferentially expressed in lymphoid tissues. Expression of schlafen genes in fibroblasts or thyoma cells either retards or ablates cell growth, indicating that the schlafen proteins probably participate in regulation of the cell cycle (Schwarz, D. A. (1998) Immunity 9:657-668).

Differential gene expression within cells is triggered in response to extracellular signals and other environmental cues. Such signals include growth factors and other mitogens such as retinoic acid; cell-cell and cell-matrix contacts; and environmental factors such as nutritional signals, toxic substances, and heat shock. Candidate genes that may play a role in differentiation can be identified by altered expression patterns upon induction of cell differentiation in vitro. For example, the REX genes display reduced expression during retinoic acid induced differentiation of murine teratocarcinoma cells (Faria et al. (1998) Mol. Cell Endocrinol. 143:155-166). The murine embryonal carcinoma cell line P19 responds to retinoic acid by differentiating into neuronal cell types. The shyc gene was isolated from differentiating P19 cells and found to be predominantly expressed in the developing and embryonic nervous system, as well as the olfactory pathway of the adult mouse brain (Koster, F. et al. (1998) Neurosci. Lett. 252:69-71). Similarly, the Bdm1 gene is upregulated during differentiation of P19 cells to neuronal cells by retinoic acid, and was widely expressed in the olfactory bulb, cerebral cortex, hippocampus, cerebellum, thalamus, and medulla oblongata (Yamauchi, Y. et al. (1999) Brain Res. Mol. Brain Res. 68:149-58). These proteins therefore appear to play a role in the differentiation and later function of neuronal cells.

The final step in cell differentiation results in a specialization that is characterized by the production of particular proteins, such as contractile proteins in muscle cells, serum proteins in liver

cells and globins in red blood cell precursors. The expression of these specialized proteins depends at least in part on cell-specific transcription factors. For example, the homobox-containing transcription factor PAX-6 is essential for early eye determination, specification of ocular tissues, and normal eye development in vertebrates. PAX-6 is also involved in regulating the expression of crystallins, the dominant structural proteins of the eye lens. Defects in crystallin proteins can cause formation of cataracts, the most common cause of visual impairment world-wide (Francis, P. J. et al. (1999) Trends Genet. 15:191-196).

In the case of epidermal differentiation, the induction of differentiation-specific genes occurs either together with or following growth arrest and is believed to be linked to the molecular events that control irreversible growth arrest. Irreversible growth arrest is an early event which occurs when cells transit from the basal to the innermost suprabasal layer of the skin and begin expressing squamous-specific genes. These genes include those involved in the formation of the cross-linked envelope, such as transglutaminase I and III, involucrin, loricin, and small proline-rich repeat (SPRR) proteins. The SPRR proteins are 8-10 kDa in molecular mass, rich in proline, glutamine, and cysteine, and contain similar repeating sequence elements. The SPRR proteins may be structural proteins with a strong secondary structure or metal-binding proteins such as metallothioneins. (Jetten, A. M. and Harvat, B. L. (1997) J. Dermatol. 24:711-725; PRINTS Entry PR00021 PRORICH Small proline-rich protein signature.)

The discovery of new proteins involved in cell differentiation and the polynucleotides encoding them satisfies a need in the art by providing new compositions which are useful in the diagnosis, prevention, and treatment of cell proliferative, developmental, and neurological disorders.

SUMMARY OF THE INVENTION

The invention features purified polypeptides, proteins involved in cell differentiation, referred to collectively as "CDIFF" and individually as "CDIFF-1," "CDIFF-2," "CDIFF-3," "CDIFF-4," "CDIFF-5," "CDIFF-6," "CDIFF-7," "CDIFF-8," "CDIFF-9," "CDIFF-10," "CDIFF-11," "CDIFF-12," "CDIFF-13," "CDIFF-14," "CDIFF-15," "CDIFF-16," "CDIFF-17," "CDIFF-18," "CDIFF-19," "CDIFF-20," "CDIFF-21," "CDIFF-22," "CDIFF-23," "CDIFF-24," "CDIFF-25," "CDIFF-26," "CDIFF-27," and "CDIFF-28." In one aspect, the invention provides an isolated polypeptide comprising an amino acid sequence selected from the group consisting of a) an amino acid sequence selected from the group consisting of SEQ ID NO:1-28, b) a naturally occurring amino acid sequence having at least 90% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NO:1-28, c) a biologically active fragment of an amino acid sequence selected from the group consisting of SEQ ID NO:1-28, and d) an immunogenic fragment of an amino acid sequence selected from the group consisting of SEQ ID NO:1-28. In one alternative, the

invention provides an isolated polypeptide comprising the amino acid sequence of SEQ ID NO:1-28.

The invention further provides an isolated polynucleotide encoding a polypeptide comprising an amino acid sequence selected from the group consisting of a) an amino acid sequence selected from the group consisting of SEQ ID NO:1-28, b) a naturally occurring amino acid sequence having at least 90% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NO:1-28, c) a biologically active fragment of an amino acid sequence selected from the group consisting of SEQ ID NO:1-28, and d) an immunogenic fragment of an amino acid sequence selected from the group consisting of SEQ ID NO:1-28. In one alternative, the polynucleotide encodes a polypeptide selected from the group consisting of SEQ ID NO:1-28. In another alternative, the polynucleotide is selected from the group consisting of SEQ ID NO:29-56.

Additionally, the invention provides a recombinant polynucleotide comprising a promoter sequence operably linked to a polynucleotide encoding a polypeptide comprising an amino acid sequence selected from the group consisting of a) an amino acid sequence selected from the group consisting of SEQ ID NO:1-28, b) a naturally occurring amino acid sequence having at least 90% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NO:1-28, c) a biologically active fragment of an amino acid sequence selected from the group consisting of SEQ ID NO:1-28, and d) an immunogenic fragment of an amino acid sequence selected from the group consisting of SEQ ID NO:1-28. In one alternative, the invention provides a cell transformed with the recombinant polynucleotide. In another alternative, the invention provides a transgenic organism comprising the recombinant polynucleotide.

The invention also provides a method for producing a polypeptide comprising an amino acid sequence selected from the group consisting of a) an amino acid sequence selected from the group consisting of SEQ ID NO:1-28, b) a naturally occurring amino acid sequence having at least 90% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NO:1-28, c) a biologically active fragment of an amino acid sequence selected from the group consisting of SEQ ID NO:1-28, and d) an immunogenic fragment of an amino acid sequence selected from the group consisting of SEQ ID NO:1-28. The method comprises a) culturing a cell under conditions suitable for expression of the polypeptide, wherein said cell is transformed with a recombinant polynucleotide comprising a promoter sequence operably linked to a polynucleotide encoding the polypeptide, and b) recovering the polypeptide so expressed.

Additionally, the invention provides an isolated antibody which specifically binds to a polypeptide comprising an amino acid sequence selected from the group consisting of a) an amino acid sequence selected from the group consisting of SEQ ID NO:1-28, b) a naturally occurring amino acid sequence having at least 90% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NO:1-28, c) a biologically active fragment of an amino acid sequence

selected from the group consisting of SEQ ID NO:1-28, and d) an immunogenic fragment of an amino acid sequence selected from the group consisting of SEQ ID NO:1-28.

The invention further provides an isolated polynucleotide comprising a polynucleotide sequence selected from the group consisting of a) a polynucleotide sequence selected from the group consisting of SEQ ID NO:29-56, b) a naturally occurring polynucleotide sequence having at least 90% sequence identity to a polynucleotide sequence selected from the group consisting of SEQ ID NO:29-56, c) a polynucleotide sequence complementary to a), d) a polynucleotide sequence complementary to b), and e) an RNA equivalent of a)-d). In one alternative, the polynucleotide comprises at least 60 contiguous nucleotides.

Additionally, the invention provides a method for detecting a target polynucleotide in a sample, said target polynucleotide having a sequence of a polynucleotide comprising a polynucleotide sequence selected from the group consisting of a) a polynucleotide sequence selected from the group consisting of SEQ ID NO:29-56, b) a naturally occurring polynucleotide sequence having at least 90% sequence identity to a polynucleotide sequence selected from the group consisting of SEQ ID NO:29-56, c) a polynucleotide sequence complementary to a), d) a polynucleotide sequence complementary to b), and e) an RNA equivalent of a)-d). The method comprises a) hybridizing the sample with a probe comprising at least 20 contiguous nucleotides comprising a sequence complementary to said target polynucleotide in the sample, and which probe specifically hybridizes to said target polynucleotide, under conditions whereby a hybridization complex is formed between said probe and said target polynucleotide or fragments thereof, and b) detecting the presence or absence of said hybridization complex, and optionally, if present, the amount thereof. In one alternative, the probe comprises at least 60 contiguous nucleotides.

The invention further provides a method for detecting a target polynucleotide in a sample, said target polynucleotide having a sequence of a polynucleotide comprising a polynucleotide sequence selected from the group consisting of a) a polynucleotide sequence selected from the group consisting of SEQ ID NO:29-56, b) a naturally occurring polynucleotide sequence having at least 90% sequence identity to a polynucleotide sequence selected from the group consisting of SEQ ID NO:29-56, c) a polynucleotide sequence complementary to a), d) a polynucleotide sequence complementary to b), and e) an RNA equivalent of a)-d). The method comprises a) amplifying said target polynucleotide or fragment thereof using polymerase chain reaction amplification, and b) detecting the presence or absence of said amplified target polynucleotide or fragment thereof, and, optionally, if present, the amount thereof.

The invention further provides a composition comprising an effective amount of a polypeptide comprising an amino acid sequence selected from the group consisting of a) an amino acid sequence selected from the group consisting of SEQ ID NO:1-28, b) a naturally occurring amino

acid sequence having at least 90% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NO:1-28, c) a biologically active fragment of an amino acid sequence selected from the group consisting of SEQ ID NO:1-28, and d) an immunogenic fragment of an amino acid sequence selected from the group consisting of SEQ ID NO:1-28, and a pharmaceutically acceptable excipient. In one embodiment, the composition comprises an amino acid sequence selected from the group consisting of SEQ ID NO:1-28. The invention additionally provides a method of treating a disease or condition associated with decreased expression of functional CDIFF, comprising administering to a patient in need of such treatment the composition.

The invention also provides a method for screening a compound for effectiveness as an agonist of a polypeptide comprising an amino acid sequence selected from the group consisting of a) an amino acid sequence selected from the group consisting of SEQ ID NO:1-28, b) a naturally occurring amino acid sequence having at least 90% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NO:1-28, c) a biologically active fragment of an amino acid sequence selected from the group consisting of SEQ ID NO:1-28, and d) an immunogenic fragment of an amino acid sequence selected from the group consisting of SEQ ID NO:1-28. The method comprises a) exposing a sample comprising the polypeptide to a compound, and b) detecting agonist activity in the sample. In one alternative, the invention provides a composition comprising an agonist compound identified by the method and a pharmaceutically acceptable excipient. In another alternative, the invention provides a method of treating a disease or condition associated with decreased expression of functional CDIFF, comprising administering to a patient in need of such treatment the composition.

Additionally, the invention provides a method for screening a compound for effectiveness as an antagonist of a polypeptide comprising an amino acid sequence selected from the group consisting of a) an amino acid sequence selected from the group consisting of SEQ ID NO:1-28, b) a naturally occurring amino acid sequence having at least 90% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NO:1-28, c) a biologically active fragment of an amino acid sequence selected from the group consisting of SEQ ID NO:1-28, and d) an immunogenic fragment of an amino acid sequence selected from the group consisting of SEQ ID NO:1-28. The method comprises a) exposing a sample comprising the polypeptide to a compound, and b) detecting antagonist activity in the sample. In one alternative, the invention provides a composition comprising an antagonist compound identified by the method and a pharmaceutically acceptable excipient. In another alternative, the invention provides a method of treating a disease or condition associated with overexpression of functional CDIFF, comprising administering to a patient in need of such treatment the composition.

The invention further provides a method of screening for a compound that specifically binds

to a polypeptide comprising an amino acid sequence selected from the group consisting of a) an amino acid sequence selected from the group consisting of SEQ ID NO:1-28, b) a naturally occurring amino acid sequence having at least 90% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NO:1-28, c) a biologically active fragment of an amino acid sequence selected from the group consisting of SEQ ID NO:1-28, and d) an immunogenic fragment of an amino acid sequence selected from the group consisting of SEQ ID NO:1-28. The method comprises a) combining the polypeptide with at least one test compound under suitable conditions, and b) detecting binding of the polypeptide to the test compound, thereby identifying a compound that specifically binds to the polypeptide.

The invention further provides a method of screening for a compound that modulates the activity of a polypeptide comprising an amino acid sequence selected from the group consisting of a) an amino acid sequence selected from the group consisting of SEQ ID NO:1-28, b) a naturally occurring amino acid sequence having at least 90% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NO:1-28, c) a biologically active fragment of an amino acid sequence selected from the group consisting of SEQ ID NO:1-28, and d) an immunogenic fragment of an amino acid sequence selected from the group consisting of SEQ ID NO:1-28. The method comprises a) combining the polypeptide with at least one test compound under conditions permissive for the activity of the polypeptide, b) assessing the activity of the polypeptide in the presence of the test compound, and c) comparing the activity of the polypeptide in the presence of the test compound with the activity of the polypeptide in the absence of the test compound, wherein a change in the activity of the polypeptide in the presence of the test compound is indicative of a compound that modulates the activity of the polypeptide.

The invention further provides a method for screening a compound for effectiveness in altering expression of a target polynucleotide, wherein said target polynucleotide comprises a sequence selected from the group consisting of SEQ ID NO:29-56, the method comprising a) exposing a sample comprising the target polynucleotide to a compound, and b) detecting altered expression of the target polynucleotide.

The invention further provides a method for assessing toxicity of a test compound, said method comprising a) treating a biological sample containing nucleic acids with the test compound; b) hybridizing the nucleic acids of the treated biological sample with a probe comprising at least 20 contiguous nucleotides of a polynucleotide comprising a polynucleotide sequence selected from the group consisting of i) a polynucleotide sequence selected from the group consisting of SEQ ID NO:29-56, ii) a naturally occurring polynucleotide sequence having at least 90% sequence identity to a polynucleotide sequence selected from the group consisting of SEQ ID NO:29-56, iii) a polynucleotide sequence complementary to i), iv) a polynucleotide sequence complementary to ii),

and v) an RNA equivalent of i)-iv). Hybridization occurs under conditions whereby a specific hybridization complex is formed between said probe and a target polynucleotide in the biological sample, said target polynucleotide comprising a polynucleotide sequence selected from the group consisting of i) a polynucleotide sequence selected from the group consisting of SEQ ID NO:29-56, ii) a naturally occurring polynucleotide sequence having at least 90% sequence identity to a polynucleotide sequence selected from the group consisting of SEQ ID NO:29-56, iii) a polynucleotide sequence complementary to i), iv) a polynucleotide sequence complementary to ii), and v) an RNA equivalent of i)-iv). Alternatively, the target polynucleotide comprises a fragment of a polynucleotide sequence selected from the group consisting of i)-v) above; c) quantifying the amount of hybridization complex; and d) comparing the amount of hybridization complex in the treated biological sample with the amount of hybridization complex in an untreated biological sample, wherein a difference in the amount of hybridization complex in the treated biological sample is indicative of toxicity of the test compound.

BRIEF DESCRIPTION OF THE TABLES

Table 1 shows polypeptide and nucleotide sequence identification numbers (SEQ ID NOs), clone identification numbers (clone IDs), cDNA libraries, and cDNA fragments used to assemble full-length sequences encoding CDIFF.

Table 2 shows features of each polypeptide sequence, including potential motifs, homologous sequences, and methods, algorithms, and searchable databases used for analysis of CDIFF.

Table 3 shows selected fragments of each nucleic acid sequence; the tissue-specific expression patterns of each nucleic acid sequence as determined by northern analysis; diseases, disorders, or conditions associated with these tissues; and the vector into which each cDNA was cloned.

Table 4 describes the tissues used to construct the cDNA libraries from which cDNA clones encoding CDIFF were isolated.

Table 5 shows the tools, programs, and algorithms used to analyze the polynucleotides and polypeptides of the invention, along with applicable descriptions, references, and threshold parameters.

DESCRIPTION OF THE INVENTION

Before the present proteins, nucleotide sequences, and methods are described, it is understood that this invention is not limited to the particular machines, materials and methods described, as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which

will be limited only by the appended claims.

It must be noted that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, a reference to "a host cell" includes a plurality of such host cells, and a reference to "an antibody" is a reference to one or more antibodies and equivalents thereof known to those skilled in the art, and so forth.

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any machines, materials, and methods similar or equivalent to those described herein can be used to practice or test the present invention, the preferred machines, materials and methods are now described. All publications mentioned herein are cited for the purpose of describing and disclosing the cell lines, protocols, reagents and vectors which are reported in the publications and which might be used in connection with the invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

DEFINITIONS

"CDIFF" refers to the amino acid sequences of substantially purified CDIFF obtained from any species, particularly a mammalian species, including bovine, ovine, porcine, murine, equine, and human, and from any source, whether natural, synthetic, semi-synthetic, or recombinant.

The term "agonist" refers to a molecule which intensifies or mimics the biological activity of CDIFF. Agonists may include proteins, nucleic acids, carbohydrates, small molecules, or any other compound or composition which modulates the activity of CDIFF either by directly interacting with CDIFF or by acting on components of the biological pathway in which CDIFF participates.

An "allelic variant" is an alternative form of the gene encoding CDIFF. Allelic variants may result from at least one mutation in the nucleic acid sequence and may result in altered mRNAs or in polypeptides whose structure or function may or may not be altered. A gene may have none, one, or many allelic variants of its naturally occurring form. Common mutational changes which give rise to allelic variants are generally ascribed to natural deletions, additions, or substitutions of nucleotides. Each of these types of changes may occur alone, or in combination with the others, one or more times in a given sequence.

"Altered" nucleic acid sequences encoding CDIFF include those sequences with deletions, insertions, or substitutions of different nucleotides, resulting in a polypeptide the same as CDIFF or a polypeptide with at least one functional characteristic of CDIFF. Included within this definition are polymorphisms which may or may not be readily detectable using a particular oligonucleotide probe of the polynucleotide encoding CDIFF, and improper or unexpected hybridization to allelic variants, with a locus other than the normal chromosomal locus for the polynucleotide sequence encoding

CDIFF. The encoded protein may also be "altered," and may contain deletions, insertions, or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent CDIFF. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues, as long as the biological or immunological activity of CDIFF is retained. For example, negatively charged amino acids may include aspartic acid and glutamic acid, and positively charged amino acids may include lysine and arginine. Amino acids with uncharged polar side chains having similar hydrophilicity values may include: asparagine and glutamine; and serine and threonine. Amino acids with uncharged side chains having similar hydrophilicity values may include: leucine, isoleucine, and valine; glycine and alanine; and phenylalanine and tyrosine.

The terms "amino acid" and "amino acid sequence" refer to an oligopeptide, peptide, polypeptide, or protein sequence, or a fragment of any of these, and to naturally occurring or synthetic molecules. Where "amino acid sequence" is recited to refer to a sequence of a naturally occurring protein molecule, "amino acid sequence" and like terms are not meant to limit the amino acid sequence to the complete native amino acid sequence associated with the recited protein molecule.

"Amplification" relates to the production of additional copies of a nucleic acid sequence. Amplification is generally carried out using polymerase chain reaction (PCR) technologies well known in the art.

The term "antagonist" refers to a molecule which inhibits or attenuates the biological activity of CDIFF. Antagonists may include proteins such as antibodies, nucleic acids, carbohydrates, small molecules, or any other compound or composition which modulates the activity of CDIFF either by directly interacting with CDIFF or by acting on components of the biological pathway in which CDIFF participates.

The term "antibody" refers to intact immunoglobulin molecules as well as to fragments thereof, such as Fab, $F(ab')_2$, and Fv fragments, which are capable of binding an epitopic determinant. Antibodies that bind CDIFF polypeptides can be prepared using intact polypeptides or using fragments containing small peptides of interest as the immunizing antigen. The polypeptide or oligopeptide used to immunize an animal (e.g., a mouse, a rat, or a rabbit) can be derived from the translation of RNA, or synthesized chemically, and can be conjugated to a carrier protein if desired. Commonly used carriers that are chemically coupled to peptides include bovine serum albumin, thyroglobulin, and keyhole limpet hemocyanin (KLH). The coupled peptide is then used to immunize the animal.

The term "antigenic determinant" refers to that region of a molecule (i.e., an epitope) that makes contact with a particular antibody. When a protein or a fragment of a protein is used to immunize a host animal, numerous regions of the protein may induce the production of antibodies

which bind specifically to antigenic determinants (particular regions or three-dimensional structures on the protein). An antigenic determinant may compete with the intact antigen (i.e., the immunogen used to elicit the immune response) for binding to an antibody.

The term "antisense" refers to any composition capable of base-pairing with the "sense" (coding) strand of a specific nucleic acid sequence. Antisense compositions may include DNA; RNA; peptide nucleic acid (PNA); oligonucleotides having modified backbone linkages such as phosphorothioates, methylphosphonates, or benzylphosphonates; oligonucleotides having modified sugar groups such as 2'-methoxyethyl sugars or 2'-methoxyethoxy sugars; or oligonucleotides having modified bases such as 5-methyl cytosine, 2'-deoxyuracil, or 7-deaza-2'-deoxyguanosine. Antisense molecules may be produced by any method including chemical synthesis or transcription. Once introduced into a cell, the complementary antisense molecule base-pairs with a naturally occurring nucleic acid sequence produced by the cell to form duplexes which block either transcription or translation. The designation "negative" or "minus" can refer to the antisense strand, and the designation "positive" or "plus" can refer to the sense strand of a reference DNA molecule.

The term "biologically active" refers to a protein having structural, regulatory, or biochemical functions of a naturally occurring molecule. Likewise, "immunologically active" or "immunogenic" refers to the capability of the natural, recombinant, or synthetic CDIFF, or of any oligopeptide thereof, to induce a specific immune response in appropriate animals or cells and to bind with specific antibodies.

"Complementary" describes the relationship between two single-stranded nucleic acid sequences that anneal by base-pairing. For example, 5'-AGT-3' pairs with its complement, 3'-TCA-5'.

A "composition comprising a given polynucleotide sequence" and a "composition comprising a given amino acid sequence" refer broadly to any composition containing the given polynucleotide or amino acid sequence. The composition may comprise a dry formulation or an aqueous solution. Compositions comprising polynucleotide sequences encoding CDIFF or fragments of CDIFF may be employed as hybridization probes. The probes may be stored in freeze-dried form and may be associated with a stabilizing agent such as a carbohydrate. In hybridizations, the probe may be deployed in an aqueous solution containing salts (e.g., NaCl), detergents (e.g., sodium dodecyl sulfate; SDS), and other components (e.g., Denhardt's solution, dry milk, salmon sperm DNA, etc.).

"Consensus sequence" refers to a nucleic acid sequence which has been subjected to repeated DNA sequence analysis to resolve uncalled bases, extended using the XL-PCR kit (PE Biosystems, Foster City CA) in the 5' and/or the 3' direction, and resequenced, or which has been assembled from one or more overlapping cDNA, EST, or genomic DNA fragments using a computer program for fragment assembly, such as the GELVIEW fragment assembly system (GCG, Madison WI) or Phrap

(University of Washington, Seattle WA). Some sequences have been both extended and assembled to produce the consensus sequence.

"Conservative amino acid substitutions" are those substitutions that are predicted to least interfere with the properties of the original protein, i.e., the structure and especially the function of the protein is conserved and not significantly changed by such substitutions. The table below shows amino acids which may be substituted for an original amino acid in a protein and which are regarded as conservative amino acid substitutions.

Original Residue	Conservative Substitution
Ala	Gly, Ser
Arg	His, Lys
Asn	Asp, Gln, His
Asp	Asn, Glu
Cys	Ala, Ser
Gln	Asn, Glu, His
Glu	Asp, Gln, His
Gly	Ala
His	Asn, Arg, Gln, Glu
Ile	Leu, Val
Leu	Ile, Val
Lys	Arg, Gln, Glu
Met	Leu, Ile
Phe	His, Met, Leu, Trp, Tyr
Ser	Cys, Thr
Thr	Ser, Val
Trp	Phe, Tyr
Tyr	His, Phe, Trp
Val	Ile, Leu, Thr

Conservative amino acid substitutions generally maintain (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a beta sheet or alpha helical conformation, (b) the charge or hydrophobicity of the molecule at the site of the substitution, and/or (c) the bulk of the side chain.

A "deletion" refers to a change in the amino acid or nucleotide sequence that results in the absence of one or more amino acid residues or nucleotides.

The term "derivative" refers to a chemically modified polynucleotide or polypeptide. Chemical modifications of a polynucleotide sequence can include, for example, replacement of hydrogen by an alkyl, acyl, hydroxyl, or amino group. A derivative polynucleotide encodes a polypeptide which retains at least one biological or immunological function of the natural molecule. A derivative polypeptide is one modified by glycosylation, pegylation, or any similar process that retains at least one biological or immunological function of the polypeptide from which it was derived.

A "detectable label" refers to a reporter molecule or enzyme that is capable of generating a

measurable signal and is covalently or noncovalently joined to a polynucleotide or polypeptide.

A "fragment" is a unique portion of CDIFF or the polynucleotide encoding CDIFF which is identical in sequence to but shorter in length than the parent sequence. A fragment may comprise up to the entire length of the defined sequence, minus one nucleotide/amino acid residue. For example, a fragment may comprise from 5 to 1000 contiguous nucleotides or amino acid residues. A fragment used as a probe, primer, antigen, therapeutic molecule, or for other purposes, may be at least 5, 10, 15, 16, 20, 25, 30, 40, 50, 60, 75, 100, 150, 250 or at least 500 contiguous nucleotides or amino acid residues in length. Fragments may be preferentially selected from certain regions of a molecule. For example, a polypeptide fragment may comprise a certain length of contiguous amino acids selected from the first 250 or 500 amino acids (or first 25% or 50% of a polypeptide) as shown in a certain defined sequence. Clearly these lengths are exemplary, and any length that is supported by the specification, including the Sequence Listing, tables, and figures, may be encompassed by the present embodiments.

A fragment of SEQ ID NO:29-56 comprises a region of unique polynucleotide sequence that specifically identifies SEQ ID NO:29-56, for example, as distinct from any other sequence in the genome from which the fragment was obtained. A fragment of SEQ ID NO:29-56 is useful, for example, in hybridization and amplification technologies and in analogous methods that distinguish SEQ ID NO:29-56 from related polynucleotide sequences. The precise length of a fragment of SEQ ID NO:29-56 and the region of SEQ ID NO:29-56 to which the fragment corresponds are routinely determinable by one of ordinary skill in the art based on the intended purpose for the fragment.

A fragment of SEQ ID NO:1-28 is encoded by a fragment of SEQ ID NO:29-56. A fragment of SEQ ID NO:1-28 comprises a region of unique amino acid sequence that specifically identifies SEQ ID NO:1-28. For example, a fragment of SEQ ID NO:1-28 is useful as an immunogenic peptide for the development of antibodies that specifically recognize SEQ ID NO:1-28. The precise length of a fragment of SEQ ID NO:1-28 and the region of SEQ ID NO:1-28 to which the fragment corresponds are routinely determinable by one of ordinary skill in the art based on the intended purpose for the fragment.

A "full-length" polynucleotide sequence is one containing at least a translation initiation codon (e.g., methionine) followed by an open reading frame and a translation termination codon. A "full-length" polynucleotide sequence encodes a "full-length" polypeptide sequence.

"Homology" refers to sequence similarity or, interchangeably, sequence identity, between two or more polynucleotide sequences or two or more polypeptide sequences.

The terms "percent identity" and "% identity," as applied to polynucleotide sequences, refer to the percentage of residue matches between at least two polynucleotide sequences aligned using a standardized algorithm. Such an algorithm may insert, in a standardized and reproducible way, gaps

in the sequences being compared in order to optimize alignment between two sequences, and therefore achieve a more meaningful comparison of the two sequences.

Percent identity between polynucleotide sequences may be determined using the default parameters of the CLUSTAL V algorithm as incorporated into the MEGALIGN version 3.12e sequence alignment program. This program is part of the LASERGENE software package, a suite of molecular biological analysis programs (DNASTAR, Madison WI). CLUSTAL V is described in Higgins, D.G. and P.M. Sharp (1989) CABIOS 5:151-153 and in Higgins, D.G. et al. (1992) CABIOS 8:189-191. For pairwise alignments of polynucleotide sequences, the default parameters are set as follows: Ktuple=2, gap penalty=5, window=4, and "diagonals saved"=4. The "weighted" residue weight table is selected as the default. Percent identity is reported by CLUSTAL V as the "percent similarity" between aligned polynucleotide sequences.

Alternatively, a suite of commonly used and freely available sequence comparison algorithms is provided by the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) (Altschul, S.F. et al. (1990) J. Mol. Biol. 215:403-410), which is available from several sources, including the NCBI, Bethesda, MD, and on the Internet at http://www.ncbi.nlm.nih.gov/BLAST/. The BLAST software suite includes various sequence analysis programs including "blastn," that is used to align a known polynucleotide sequence with other polynucleotide sequences from a variety of databases. Also available is a tool called "BLAST 2 Sequences" that is used for direct pairwise comparison of two nucleotide sequences. "BLAST 2 Sequences" can be accessed and used interactively at http://www.ncbi.nlm.nih.gov/gorf/bl2.html. The "BLAST 2 Sequences" tool can be used for both blastn and blastp (discussed below). BLAST programs are commonly used with gap and other parameters set to default settings. For example, to compare two nucleotide sequences, one may use blastn with the "BLAST 2 Sequences" tool Version 2.0.12 (April-21-2000) set at default parameters. Such default parameters may be, for example:

Matrix: BLOSUM62
Reward for match: 1
Penalty for mismatch: -2
Open Gap: 5 and Extension Gap: 2 penalties
Gap x drop-off: 50
Expect: 10
Word Size: 11
Filter: on

Percent identity may be measured over the length of an entire defined sequence, for example, as defined by a particular SEQ ID number, or may be measured over a shorter length, for example, over the length of a fragment taken from a larger, defined sequence, for instance, a fragment of at

least 20, at least 30, at least 40, at least 50, at least 70, at least 100, or at least 200 contiguous nucleotides. Such lengths are exemplary only, and it is understood that any fragment length supported by the sequences shown herein, in the tables, figures, or Sequence Listing, may be used to describe a length over which percentage identity may be measured.

Nucleic acid sequences that do not show a high degree of identity may nevertheless encode similar amino acid sequences due to the degeneracy of the genetic code. It is understood that changes in a nucleic acid sequence can be made using this degeneracy to produce multiple nucleic acid sequences that all encode substantially the same protein.

The phrases "percent identity" and "% identity," as applied to polypeptide sequences, refer to the percentage of residue matches between at least two polypeptide sequences aligned using a standardized algorithm. Methods of polypeptide sequence alignment are well-known. Some alignment methods take into account conservative amino acid substitutions. Such conservative substitutions, explained in more detail above, generally preserve the charge and hydrophobicity at the site of substitution, thus preserving the structure (and therefore function) of the polypeptide.

Percent identity between polypeptide sequences may be determined using the default parameters of the CLUSTAL V algorithm as incorporated into the MEGALIGN version 3.12e sequence alignment program (described and referenced above). For pairwise alignments of polypeptide sequences using CLUSTAL V, the default parameters are set as follows: Ktuple=1, gap penalty=3, window=5, and "diagonals saved"=5. The PAM250 matrix is selected as the default residue weight table. As with polynucleotide alignments, the percent identity is reported by CLUSTAL V as the "percent similarity" between aligned polypeptide sequence pairs.

Alternatively the NCBI BLAST software suite may be used. For example, for a pairwise comparison of two polypeptide sequences, one may use the "BLAST 2 Sequences" tool Version 2.0.12 (Apr-21-2000) with blastp set at default parameters. Such default parameters may be, for example:

Matrix: BLOSUM62

Open Gap: 11 and Extension Gap: 1 penalties

Gap x drop-off: 50

Expect: 10

Word Size: 3

Filter: on

Percent identity may be measured over the length of an entire defined polypeptide sequence, for example, as defined by a particular SEQ ID number, or may be measured over a shorter length, for example, over the length of a fragment taken from a larger, defined polypeptide sequence, for instance, a fragment of at least 15, at least 20, at least 30, at least 40, at least 50, at least 70 or at least

150 contiguous residues. Such lengths are exemplary only, and it is understood that any fragment length supported by the sequences shown herein, in the tables, figures or Sequence Listing, may be used to describe a length over which percentage identity may be measured.

"Human artificial chromosomes" (HACs) are linear microchromosomes which may contain DNA sequences of about 6 kb to 10 Mb in size, and which contain all of the elements required for chromosome replication, segregation and maintenance.

The term "humanized antibody" refers to an antibody molecule in which the amino acid sequence in the non-antigen binding regions has been altered so that the antibody more closely resembles a human antibody, and still retains its original binding ability.

"Hybridization" refers to the process by which a polynucleotide strand anneals with a complementary strand through base pairing under defined hybridization conditions. Specific hybridization is an indication that two nucleic acid sequences share a high degree of complementarity. Specific hybridization complexes form under permissive annealing conditions and remain hybridized after the "washing" step(s). The washing step(s) is particularly important in determining the stringency of the hybridization process, with more stringent conditions allowing less non-specific binding, i.e., binding between pairs of nucleic acid strands that are not perfectly matched. Permissive conditions for annealing of nucleic acid sequences are routinely determinable by one of ordinary skill in the art and may be consistent among hybridization experiments, whereas wash conditions may be varied among experiments to achieve the desired stringency, and therefore hybridization specificity. Permissive annealing conditions occur, for example, at 68°C in the presence of about 6 x SSC, about 1% (w/v) SDS, and about 100 μg/ml sheared, denatured salmon sperm DNA.

Generally, stringency of hybridization is expressed, in part, with reference to the temperature under which the wash step is carried out. Such wash temperatures are typically selected to be about 5°C to 20°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. An equation for calculating T_m and conditions for nucleic acid hybridization are well known and can be found in Sambrook, J. et al., 1989, Molecular Cloning: A Laboratory Manual, 2nd ed., vol. 1-3, Cold Spring Harbor Press, Plainview NY; specifically see volume 2, chapter 9.

High stringency conditions for hybridization between polynucleotides of the present invention include wash conditions of 68°C in the presence of about 0.2 x SSC and about 0.1% SDS, for 1 hour. Alternatively, temperatures of about 65°C, 60°C, 55°C, or 42°C may be used. SSC concentration may be varied from about 0.1 to 2 x SSC, with SDS being present at about 0.1%. Typically, blocking reagents are used to block non-specific hybridization. Such blocking reagents include, for instance, sheared and denatured salmon sperm DNA at about 100-200 μg/ml. Organic

solvent, such as formamide at a concentration of about 35-50% v/v, may also be used under particular circumstances, such as for RNA:DNA hybridizations. Useful variations on these wash conditions will be readily apparent to those of ordinary skill in the art. Hybridization, particularly under high stringency conditions, may be suggestive of evolutionary similarity between the nucleotides. Such similarity is strongly indicative of a similar role for the nucleotides and their encoded polypeptides.

The term "hybridization complex" refers to a complex formed between two nucleic acid sequences by virtue of the formation of hydrogen bonds between complementary bases. A hybridization complex may be formed in solution (e.g., C_0t or R_0t analysis) or formed between one nucleic acid sequence present in solution and another nucleic acid sequence immobilized on a solid support (e.g., paper, membranes, filters, chips, pins or glass slides, or any other appropriate substrate to which cells or their nucleic acids have been fixed).

The words "insertion" and "addition" refer to changes in an amino acid or nucleotide sequence resulting in the addition of one or more amino acid residues or nucleotides, respectively.

"Immune response" can refer to conditions associated with inflammation, trauma, immune disorders, or infectious or genetic disease, etc. These conditions can be characterized by expression of various factors, e.g., cytokines, chemokines, and other signaling molecules, which may affect cellular and systemic defense systems.

An "immunogenic fragment" is a polypeptide or oligopeptide fragment of CDIFF which is capable of eliciting an immune response when introduced into a living organism, for example, a mammal. The term "immunogenic fragment" also includes any polypeptide or oligopeptide fragment of CDIFF which is useful in any of the antibody production methods disclosed herein or known in the art.

The term "microarray" refers to an arrangement of a plurality of polynucleotides, polypeptides, or other chemical compounds on a substrate.

The terms "element" and "array element" refer to a polynucleotide, polypeptide, or other chemical compound having a unique and defined position on a microarray.

The term "modulate" refers to a change in the activity of CDIFF. For example, modulation may cause an increase or a decrease in protein activity, binding characteristics, or any other biological, functional, or immunological properties of CDIFF.

The phrases "nucleic acid" and "nucleic acid sequence" refer to a nucleotide, oligonucleotide, polynucleotide, or any fragment thereof. These phrases also refer to DNA or RNA of genomic or synthetic origin which may be single-stranded or double-stranded and may represent the sense or the antisense strand, to peptide nucleic acid (PNA), or to any DNA-like or RNA-like material.

"Operably linked" refers to the situation in which a first nucleic acid sequence is placed in a functional relationship with a second nucleic acid sequence. For instance, a promoter is operably

linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Operably linked DNA sequences may be in close proximity or contiguous and, where necessary to join two protein coding regions, in the same reading frame.

"Peptide nucleic acid" (PNA) refers to an antisense molecule or anti-gene agent which comprises an oligonucleotide of at least about 5 nucleotides in length linked to a peptide backbone of amino acid residues ending in lysine. The terminal lysine confers solubility to the composition. PNAs preferentially bind complementary single stranded DNA or RNA and stop transcript elongation, and may be pegylated to extend their lifespan in the cell.

"Post-translational modification" of an CDIFF may involve lipidation, glycosylation, phosphorylation, acetylation, racemization, proteolytic cleavage, and other modifications known in the art. These processes may occur synthetically or biochemically. Biochemical modifications will vary by cell type depending on the enzymatic milieu of CDIFF.

"Probe" refers to nucleic acid sequences encoding CDIFF, their complements, or fragments thereof, which are used to detect identical, allelic or related nucleic acid sequences. Probes are isolated oligonucleotides or polynucleotides attached to a detectable label or reporter molecule. Typical labels include radioactive isotopes, ligands, chemiluminescent agents, and enzymes. "Primers" are short nucleic acids, usually DNA oligonucleotides, which may be annealed to a target polynucleotide by complementary base-pairing. The primer may then be extended along the target DNA strand by a DNA polymerase enzyme. Primer pairs can be used for amplification (and identification) of a nucleic acid sequence, e.g., by the polymerase chain reaction (PCR).

Probes and primers as used in the present invention typically comprise at least 15 contiguous nucleotides of a known sequence. In order to enhance specificity, longer probes and primers may also be employed, such as probes and primers that comprise at least 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, or at least 150 consecutive nucleotides of the disclosed nucleic acid sequences. Probes and primers may be considerably longer than these examples, and it is understood that any length supported by the specification, including the tables, figures, and Sequence Listing, may be used.

Methods for preparing and using probes and primers are described in the references, for example Sambrook, J. et al. (1989) Molecular Cloning: A Laboratory Manual, 2^{nd} ed., vol. 1-3, Cold Spring Harbor Press, Plainview NY; Ausubel, F.M. et al. (1987) Current Protocols in Molecular Biology, Greene Publ. Assoc. & Wiley-Intersciences, New York NY; Innis, M. et al. (1990) PCR Protocols, A Guide to Methods and Applications, Academic Press, San Diego CA. PCR primer pairs can be derived from a known sequence, for example, by using computer programs intended for that purpose such as Primer (Version 0.5, 1991, Whitehead Institute for Biomedical Research, Cambridge MA).

Oligonucleotides for use as primers are selected using software known in the art for such

purpose. For example, OLIGO 4.06 software is useful for the selection of PCR primer pairs of up to 100 nucleotides each, and for the analysis of oligonucleotides and larger polynucleotides of up to 5,000 nucleotides from an input polynucleotide sequence of up to 32 kilobases. Similar primer selection programs have incorporated additional features for expanded capabilities. For example, the PrimOU primer selection program (available to the public from the Genome Center at University of Texas South West Medical Center, Dallas TX) is capable of choosing specific primers from megabase sequences and is thus useful for designing primers on a genome-wide scope. The Primer3 primer selection program (available to the public from the Whitehead Institute/MIT Center for Genome Research, Cambridge MA) allows the user to input a "mispriming library," in which sequences to avoid as primer binding sites are user-specified. Primer3 is useful, in particular, for the selection of oligonucleotides for microarrays. (The source code for the latter two primer selection programs may also be obtained from their respective sources and modified to meet the user's specific needs.) The PrimeGen program (available to the public from the UK Human Genome Mapping Project Resource Centre, Cambridge UK) designs primers based on multiple sequence alignments, thereby allowing selection of primers that hybridize to either the most conserved or least conserved regions of aligned nucleic acid sequences. Hence, this program is useful for identification of both unique and conserved oligonucleotides and polynucleotide fragments. The oligonucleotides and polynucleotide fragments identified by any of the above selection methods are useful in hybridization technologies, for example, as PCR or sequencing primers, microarray elements, or specific probes to identify fully or partially complementary polynucleotides in a sample of nucleic acids. Methods of oligonucleotide selection are not limited to those described above.

A "recombinant nucleic acid" is a sequence that is not naturally occurring or has a sequence that is made by an artificial combination of two or more otherwise separated segments of sequence. This artificial combination is often accomplished by chemical synthesis or, more commonly, by the artificial manipulation of isolated segments of nucleic acids, e.g., by genetic engineering techniques such as those described in Sambrook, supra. The term recombinant includes nucleic acids that have been altered solely by addition, substitution, or deletion of a portion of the nucleic acid. Frequently, a recombinant nucleic acid may include a nucleic acid sequence operably linked to a promoter sequence. Such a recombinant nucleic acid may be part of a vector that is used, for example, to transform a cell.

Alternatively, such recombinant nucleic acids may be part of a viral vector, e.g., based on a vaccinia virus, that could be use to vaccinate a mammal wherein the recombinant nucleic acid is expressed, inducing a protective immunological response in the mammal.

A "regulatory element" refers to a nucleic acid sequence usually derived from untranslated regions of a gene and includes enhancers, promoters, introns, and 5' and 3' untranslated regions

(UTRs). Regulatory elements interact with host or viral proteins which control transcription, translation, or RNA stability.

"Reporter molecules" are chemical or biochemical moieties used for labeling a nucleic acid, amino acid, or antibody. Reporter molecules include radionuclides; enzymes; fluorescent, chemiluminescent, or chromogenic agents; substrates; cofactors; inhibitors; magnetic particles; and other moieties known in the art.

An "RNA equivalent," in reference to a DNA sequence, is composed of the same linear sequence of nucleotides as the reference DNA sequence with the exception that all occurrences of the nitrogenous base thymine are replaced with uracil, and the sugar backbone is composed of ribose instead of deoxyribose.

The term "sample" is used in its broadest sense. A sample suspected of containing nucleic acids encoding CDIFF, or fragments thereof, or CDIFF itself, may comprise a bodily fluid; an extract from a cell, chromosome, organelle, or membrane isolated from a cell; a cell; genomic DNA, RNA, or cDNA, in solution or bound to a substrate; a tissue; a tissue print; etc.

The terms "specific binding" and "specifically binding" refer to that interaction between a protein or peptide and an agonist, an antibody, an antagonist, a small molecule, or any natural or synthetic binding composition. The interaction is dependent upon the presence of a particular structure of the protein, e.g., the antigenic determinant or epitope, recognized by the binding molecule. For example, if an antibody is specific for epitope "A," the presence of a polypeptide comprising the epitope A, or the presence of free unlabeled A, in a reaction containing free labeled A and the antibody will reduce the amount of labeled A that binds to the antibody.

The term "substantially purified" refers to nucleic acid or amino acid sequences that are removed from their natural environment and are isolated or separated, and are at least 60% free, preferably at least 75% free, and most preferably at least SEQ ID NO:29-56 free from other components with which they are naturally associated.

A "substitution" refers to the replacement of one or more amino acid residues or nucleotides by different amino acid residues or nucleotides, respectively.

"Substrate" refers to any suitable rigid or semi-rigid support including membranes, filters, chips, slides, wafers, fibers, magnetic or nonmagnetic beads, gels, tubing, plates, polymers, microparticles and capillaries. The substrate can have a variety of surface forms, such as wells, trenches, pins, channels and pores, to which polynucleotides or polypeptides are bound.

A "transcript image" refers to the collective pattern of gene expression by a particular cell type or tissue under given conditions at a given time.

"Transformation" describes a process by which exogenous DNA is introduced into a recipient cell. Transformation may occur under natural or artificial conditions according to various methods

well known in the art, and may rely on any known method for the insertion of foreign nucleic acid sequences into a prokaryotic or eukaryotic host cell. The method for transformation is selected based on the type of host cell being transformed and may include, but is not limited to, bacteriophage or viral infection, electroporation, heat shock, lipofection, and particle bombardment. The term "transformed" cells includes stably transformed cells in which the inserted DNA is capable of replication either as an autonomously replicating plasmid or as part of the host chromosome, as well as transiently transformed cells which express the inserted DNA or RNA for limited periods of time.

A "transgenic organism," as used herein, is any organism, including but not limited to animals and plants, in which one or more of the cells of the organism contains heterologous nucleic acid introduced by way of human intervention, such as by transgenic techniques well known in the art. The nucleic acid is introduced into the cell, directly or indirectly by introduction into a precursor of the cell, by way of deliberate genetic manipulation, such as by microinjection or by infection with a recombinant virus. The term genetic manipulation does not include classical cross-breeding, or in vitro fertilization, but rather is directed to the introduction of a recombinant DNA molecule. The transgenic organisms contemplated in accordance with the present invention include bacteria, cyanobacteria, fungi, plants, and animals. The isolated DNA of the present invention can be introduced into the host by methods known in the art, for example infection, transfection, transformation or transconjugation. Techniques for transferring the DNA of the present invention into such organisms are widely known and provided in references such as Sambrook, J. et al. (1989), supra.

A "variant" of a particular nucleic acid sequence is defined as a nucleic acid sequence having at least 40% sequence identity to the particular nucleic acid sequence over a certain length of one of the nucleic acid sequences using blastn with the "BLAST 2 Sequences" tool Version 2.0.9 (May-07-1999) set at default parameters. Such a pair of nucleic acids may show, for example, at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95% or at least 98% or greater sequence identity over a certain defined length. A variant may be described as, for example, an "allelic" (as defined above), "splice," "species," or "polymorphic" variant. A splice variant may have significant identity to a reference molecule, but will generally have a greater or lesser number of polynucleotides due to alternative splicing of exons during mRNA processing. The corresponding polypeptide may possess additional functional domains or lack domains that are present in the reference molecule. Species variants are polynucleotide sequences that vary from one species to another. The resulting polypeptides generally will have significant amino acid identity relative to each other. A polymorphic variant is a variation in the polynucleotide sequence of a particular gene between individuals of a given species. Polymorphic variants also may encompass "single nucleotide polymorphisms" (SNPs) in which the polynucleotide sequence varies by one nucleotide base. The

presence of SNPs may be indicative of, for example, a certain population, a disease state, or a propensity for a disease state.

A "variant" of a particular polypeptide sequence is defined as a polypeptide sequence having at least 40% sequence identity to the particular polypeptide sequence over a certain length of one of the polypeptide sequences using blastp with the "BLAST 2 Sequences" tool Version 2.0.9 (May-07-1999) set at default parameters. Such a pair of polypeptides may show, for example, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 98% or greater sequence identity over a certain defined length of one of the polypeptides.

THE INVENTION

The invention is based on the discovery of new human proteins involved in cell differentiation (CDIFF), the polynucleotides encoding CDIFF, and the use of these compositions for the diagnosis, treatment, or prevention of cell proliferative, developmental, and neurological disorders.

Table 1 lists the Incyte clones used to assemble full length nucleotide sequences encoding CDIFF. Columns 1 and 2 show the sequence identification numbers (SEQ ID NOs) of the polypeptide and nucleotide sequences, respectively. Column 3 shows the clone IDs of the Incyte clones in which nucleic acids encoding each CDIFF were identified, and column 4 shows the cDNA libraries from which these clones were isolated. Column 5 shows Incyte clones and their corresponding cDNA libraries. Clones for which cDNA libraries are not indicated were derived from pooled cDNA libraries. In some cases, GenBank sequence identifiers are also shown in column 5. The Incyte clones and GenBank cDNA sequences, where indicated, in column 5 were used to assemble the consensus nucleotide sequence of each CDIFF and are useful as fragments in hybridization technologies.

The columns of Table 2 show various properties of each of the polypeptides of the invention: column 1 references the SEQ ID NO; column 2 shows the number of amino acid residues in each polypeptide; column 3 shows potential phosphorylation sites; column 4 shows potential glycosylation sites; column 5 shows the amino acid residues comprising signature sequences and motifs; column 6 shows homologous sequences as identified by BLAST analysis along with relevant citations, all of which are expressly incorporated by reference herein in their entirety; and column 7 shows analytical methods and in some cases, searchable databases to which the analytical methods were applied. The methods of column 7 were used to characterize each polypeptide through sequence homology and protein motifs.

The columns of Table 3 show the tissue-specificity and diseases, disorders, or conditions associated with nucleotide sequences encoding CDIFF. The first column of Table 3 lists the nucleotide SEQ ID NOs. Column 2 lists fragments of the nucleotide sequences of column 1. These

fragments are useful, for example, in hybridization or amplification technologies to identify SEQ ID NO:29-56 and to distinguish between SEQ ID NO:29-56 and related polynucleotide sequences. The polypeptides encoded by these fragments are useful, for example, as immunogenic peptides. Column 3 lists tissue categories which express CDIFF as a fraction of total tissues expressing CDIFF. Column 4 lists diseases, disorders, or conditions associated with those tissues expressing CDIFF as a fraction of total tissues expressing CDIFF. Column 5 lists the vectors used to subclone each cDNA library.

The columns of Table 4 show descriptions of the tissues used to construct the cDNA libraries from which cDNA clones encoding CDIFF were isolated. Column 1 references the nucleotide SEQ ID NOs, column 2 shows the cDNA libraries from which these clones were isolated, and column 3 shows the tissue origins and other descriptive information relevant to the cDNA libraries in column 2.

SEQ ID NO:32 maps to chromosome 1 within the interval from 152.2 to 157.4 centiMorgans, to chromosome 3 within the interval from 157.4 to 158.0 centiMorgans, and to the X chromosome within the interval from 104.9 to 150.3 centiMorgans. The interval on chromosome 1 from 152.2 to 157.4 centiMorgans also contains genes associated with leukemia, hypothyroidism, and adrenal hyperplasia. The interval on the X chromosome from 104.9 to 150.3 centiMorgans also contains genes associated with X-linked lissencephaly, leiomyomatosis with Alport syndrome, lymphoproliferative syndrome, Bruton agammaglobulinemia, and diffuse angiokeratoma. SEQ ID NO:37 maps to chromosome 11 within the interval from 19.6 to 23.2 centiMorgans. SEQ ID NO:39 maps to chromosome 16 within the interval from 109.1 to 130.8 centiMorgans, and to chromosome 22 within the interval from 45.5 to 58.1 centiMorgans. The interval on chromosome 16 from 109.1 to 130.8 centiMorgans also contains a gene associated with gastric cancer susceptibility. SEQ ID NO:45 maps to chromosome 7 within the interval from 105.2 to 109.0 centiMorgans, to chromosome 17 within the interval from 65.0 to 90.2 centiMorgans, and to chromosome 20 within the interval from 50.2 to 54.9 centiMorgans. The interval on chromosome 7 from 105.2 to 109.0 centiMorgans also contains a gene associated with osteogenesis imperfecta. The interval on chromosome 17 from 65.0 to 90.2 centiMorgans also contains genes associated with breast cancer, hepatic leukemia, myeloperoxidase deficiency, muscular dystrophy, periodic paralysis, and placental growth. SEQ ID NO:54 maps to chromosome 12 within the interval from 21.3 to 36.1 centiMorgans. SEQ ID NO:55 maps to chromosome 1 within the interval from 22.9 to 39.9 centiMorgans and to chromosome 3 within the interval from 30.9 to 43.0 centiMorgans.

The invention also encompasses CDIFF variants. A preferred CDIFF variant is one which has at least about 80%, or alternatively at least about 90%, or even at least about 95% amino acid sequence identity to the CDIFF amino acid sequence, and which contains at least one functional or structural characteristic of CDIFF.

The invention also encompasses polynucleotides which encode CDIFF. In a particular embodiment, the invention encompasses a polynucleotide sequence comprising a sequence selected from the group consisting of SEQ ID NO:29-56, which encodes CDIFF. The polynucleotide sequences of SEQ ID NO:29-56, as presented in the Sequence Listing, embrace the equivalent RNA sequences, wherein occurrences of the nitrogenous base thymine are replaced with uracil, and the sugar backbone is composed of ribose instead of deoxyribose.

The invention also encompasses a variant of a polynucleotide sequence encoding CDIFF. In particular, such a variant polynucleotide sequence will have at least about 80%, or alternatively at least about 90%, or even at least about 95% polynucleotide sequence identity to the polynucleotide sequence encoding CDIFF. A particular aspect of the invention encompasses a variant of a polynucleotide sequence comprising a sequence selected from the group consisting of SEQ ID NO:29-56 which has at least about 80%, or alternatively at least about 90%, or even at least about 95% polynucleotide sequence identity to a nucleic acid sequence selected from the group consisting of SEQ ID NO:29-56. Any one of the polynucleotide variants described above can encode an amino acid sequence which contains at least one functional or structural characteristic of CDIFF.

It will be appreciated by those skilled in the art that as a result of the degeneracy of the genetic code, a multitude of polynucleotide sequences encoding CDIFF, some bearing minimal similarity to the polynucleotide sequences of any known and naturally occurring gene, may be produced. Thus, the invention contemplates each and every possible variation of polynucleotide sequence that could be made by selecting combinations based on possible codon choices. These combinations are made in accordance with the standard triplet genetic code as applied to the polynucleotide sequence of naturally occurring CDIFF, and all such variations are to be considered as being specifically disclosed.

Although nucleotide sequences which encode CDIFF and its variants are generally capable of hybridizing to the nucleotide sequence of the naturally occurring CDIFF under appropriately selected conditions of stringency, it may be advantageous to produce nucleotide sequences encoding CDIFF or its derivatives possessing a substantially different codon usage, e.g., inclusion of non-naturally occurring codons. Codons may be selected to increase the rate at which expression of the peptide occurs in a particular prokaryotic or eukaryotic host in accordance with the frequency with which particular codons are utilized by the host. Other reasons for substantially altering the nucleotide sequence encoding CDIFF and its derivatives without altering the encoded amino acid sequences include the production of RNA transcripts having more desirable properties, such as a greater half-life, than transcripts produced from the naturally occurring sequence.

The invention also encompasses production of DNA sequences which encode CDIFF and CDIFF derivatives, or fragments thereof, entirely by synthetic chemistry. After production, the

synthetic sequence may be inserted into any of the many available expression vectors and cell systems using reagents well known in the art. Moreover, synthetic chemistry may be used to introduce mutations into a sequence encoding CDIFF or any fragment thereof.

Also encompassed by the invention are polynucleotide sequences that are capable of hybridizing to the claimed polynucleotide sequences, and, in particular, to those shown in SEQ ID NO:29-56 and fragments thereof under various conditions of stringency. (See, e.g., Wahl, G.M. and S.L. Berger (1987) Methods Enzymol. 152:399-407; Kimmel, A.R. (1987) Methods Enzymol. 152:507-511.) Hybridization conditions, including annealing and wash conditions, are described in "Definitions."

Methods for DNA sequencing are well known in the art and may be used to practice any of the embodiments of the invention. The methods may employ such enzymes as the Klenow fragment of DNA polymerase I, SEQUENASE (US Biochemical, Cleveland OH), Taq polymerase (PE Biosystems, Foster City CA), thermostable T7 polymerase (Amersham Pharmacia Biotech, Piscataway NJ), or combinations of polymerases and proofreading exonucleases such as those found in the ELONGASE amplification system (Life Technologies, Gaithersburg MD). Preferably, sequence preparation is automated with machines such as the MICROLAB 2200 liquid transfer system (Hamilton, Reno NV), PTC200 thermal cycler (MJ Research, Watertown MA) and ABI CATALYST 800 thermal cycler (PE Biosystems). Sequencing is then carried out using either the ABI 373 or 377 DNA sequencing system (PE Biosystems), the MEGABACE 1000 DNA sequencing system (Molecular Dynamics, Sunnyvale CA), or other systems known in the art. The resulting sequences are analyzed using a variety of algorithms which are well known in the art. (See, e.g., Ausubel, F.M. (1997) Short Protocols in Molecular Biology, John Wiley & Sons, New York NY, unit 7.7; Meyers, R.A. (1995) Molecular Biology and Biotechnology, Wiley VCH, New York NY, pp. 856-853.)

The nucleic acid sequences encoding CDIFF may be extended utilizing a partial nucleotide sequence and employing various PCR-based methods known in the art to detect upstream sequences, such as promoters and regulatory elements. For example, one method which may be employed, restriction-site PCR, uses universal and nested primers to amplify unknown sequence from genomic DNA within a cloning vector. (See, e.g., Sarkar, G. (1993) PCR Methods Applic. 2:318-322.) Another method, inverse PCR, uses primers that extend in divergent directions to amplify unknown sequence from a circularized template. The template is derived from restriction fragments comprising a known genomic locus and surrounding sequences. (See, e.g., Triglia, T. et al. (1988) Nucleic Acids Res. 16:8186.) A third method, capture PCR, involves PCR amplification of DNA fragments adjacent to known sequences in human and yeast artificial chromosome DNA. (See, e.g., Lagerstrom, M. et al. (1991) PCR Methods Applic. 1:111-119.) In this method, multiple restriction enzyme

digestions and ligations may be used to insert an engineered double-stranded sequence into a region of unknown sequence before performing PCR. Other methods which may be used to retrieve unknown sequences are known in the art. (See, e.g., Parker, J.D. et al. (1991) Nucleic Acids Res. 19:3055-3060). Additionally, one may use PCR, nested primers, and PROMOTERFINDER libraries (Clontech, Palo Alto CA) to walk genomic DNA. This procedure avoids the need to screen libraries and is useful in finding intron/exon junctions. For all PCR-based methods, primers may be designed using commercially available software, such as OLIGO 4.06 Primer Analysis software (National Biosciences, Plymouth MN) or another appropriate program, to be about 22 to 30 nucleotides in length, to have a GC content of about 50% or more, and to anneal to the template at temperatures of about 68°C to 72°C.

When screening for full-length cDNAs, it is preferable to use libraries that have been size-selected to include larger cDNAs. In addition, random-primed libraries, which often include sequences containing the 5' regions of genes, are preferable for situations in which an oligo d(T) library does not yield a full-length cDNA. Genomic libraries may be useful for extension of sequence into 5' non-transcribed regulatory regions.

Capillary electrophoresis systems which are commercially available may be used to analyze the size or confirm the nucleotide sequence of sequencing or PCR products. In particular, capillary sequencing may employ flowable polymers for electrophoretic separation, four different nucleotide-specific, laser-stimulated fluorescent dyes, and a charge coupled device camera for detection of the emitted wavelengths. Output/light intensity may be converted to electrical signal using appropriate software (e.g., GENOTYPER and SEQUENCE NAVIGATOR, PE Biosystems), and the entire process from loading of samples to computer analysis and electronic data display may be computer controlled. Capillary electrophoresis is especially preferable for sequencing small DNA fragments which may be present in limited amounts in a particular sample.

In another embodiment of the invention, polynucleotide sequences or fragments thereof which encode CDIFF may be cloned in recombinant DNA molecules that direct expression of CDIFF, or fragments or functional equivalents thereof, in appropriate host cells. Due to the inherent degeneracy of the genetic code, other DNA sequences which encode substantially the same or a functionally equivalent amino acid sequence may be produced and used to express CDIFF.

The nucleotide sequences of the present invention can be engineered using methods generally known in the art in order to alter CDIFF-encoding sequences for a variety of purposes including, but not limited to, modification of the cloning, processing, and/or expression of the gene product. DNA shuffling by random fragmentation and PCR reassembly of gene fragments and synthetic oligonucleotides may be used to engineer the nucleotide sequences. For example, oligonucleotide-mediated site-directed mutagenesis may be used to introduce mutations that create new restriction

sites, alter glycosylation patterns, change codon preference, produce splice variants, and so forth.

The nucleotides of the present invention may be subjected to DNA shuffling techniques such as MOLECULARBREEDING (Maxygen Inc., Santa Clara CA; described in U.S. Patent Number 5,837,458; Chang, C.-C. et al. (1999) Nat. Biotechnol. 17:793-797; Christians, F.C. et al. (1999) Nat. Biotechnol. 17:259-264; and Crameri, A. et al. (1996) Nat. Biotechnol. 14:315-319) to alter or improve the biological properties of CDIFF, such as its biological or enzymatic activity or its ability to bind to other molecules or compounds. DNA shuffling is a process by which a library of gene variants is produced using PCR-mediated recombination of gene fragments. The library is then subjected to selection or screening procedures that identify those gene variants with the desired properties. These preferred variants may then be pooled and further subjected to recursive rounds of DNA shuffling and selection/screening. Thus, genetic diversity is created through "artificial" breeding and rapid molecular evolution. For example, fragments of a single gene containing random point mutations may be recombined, screened, and then reshuffled until the desired properties are optimized. Alternatively, fragments of a given gene may be recombined with fragments of homologous genes in the same gene family, either from the same or different species, thereby maximizing the genetic diversity of multiple naturally occurring genes in a directed and controllable manner.

In another embodiment, sequences encoding CDIFF may be synthesized, in whole or in part, using chemical methods well known in the art. (See, e.g., Caruthers, M.H. et al. (1980) Nucleic Acids Symp. Ser. 7:215-223; Horn, T. et al. (1980) Nucleic Acids Symp. Ser. 7:225-232.) Alternatively, CDIFF itself or a fragment thereof may be synthesized using chemical methods. For example, peptide synthesis can be performed using various solution-phase or solid-phase techniques. (See, e.g., Creighton, T. (1984) Proteins, Structures and Molecular Properties, WH Freeman, New York NY, pp. 55-60; and Roberge, J.Y. et al. (1995) Science 269:202-204.) Automated synthesis may be achieved using the ABI 431A peptide synthesizer (PE Biosystems). Additionally, the amino acid sequence of CDIFF, or any part thereof, may be altered during direct synthesis and/or combined with sequences from other proteins, or any part thereof, to produce a variant polypeptide or a polypeptide having a sequence of a naturally occurring polypeptide.

The peptide may be substantially purified by preparative high performance liquid chromatography. (See, e.g., Chiez, R.M. and F.Z. Regnier (1990) Methods Enzymol. 182:392-421.) The composition of the synthetic peptides may be confirmed by amino acid analysis or by sequencing. (See, e.g., Creighton, supra, pp. 28-53.)

In order to express a biologically active CDIFF, the nucleotide sequences encoding CDIFF or derivatives thereof may be inserted into an appropriate expression vector, i.e., a vector which contains the necessary elements for transcriptional and translational control of the inserted coding sequence in

a suitable host. These elements include regulatory sequences, such as enhancers, constitutive and inducible promoters, and 5'and 3'untranslated regions in the vector and in polynucleotide sequences encoding CDIFF. Such elements may vary in their strength and specificity. Specific initiation signals may also be used to achieve more efficient translation of sequences encoding CDIFF. Such signals include the ATG initiation codon and adjacent sequences, e.g. the Kozak sequence. In cases where sequences encoding CDIFF and its initiation codon and upstream regulatory sequences are inserted into the appropriate expression vector, no additional transcriptional or translational control signals may be needed. However, in cases where only coding sequence, or a fragment thereof, is inserted, exogenous translational control signals including an in-frame ATG initiation codon should be provided by the vector. Exogenous translational elements and initiation codons may be of various origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of enhancers appropriate for the particular host cell system used. (See, e.g., Scharf, D. et al. (1994) Results Probl. Cell Differ. 20:125-162.)

Methods which are well known to those skilled in the art may be used to construct expression vectors containing sequences encoding CDIFF and appropriate transcriptional and translational control elements. These methods include in vitro recombinant DNA techniques, synthetic techniques, and in vivo genetic recombination. (See, e.g., Sambrook, J. et al. (1989) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, Plainview NY, ch. 4, 8, and 16-17; Ausubel, F.M. et al. (1995) Current Protocols in Molecular Biology, John Wiley & Sons, New York NY, ch. 9, 13, and 16.)

A variety of expression vector/host systems may be utilized to contain and express sequences encoding CDIFF. These include, but are not limited to, microorganisms such as bacteria transformed with recombinant bacteriophage, plasmid, or cosmid DNA expression vectors; yeast transformed with yeast expression vectors; insect cell systems infected with viral expression vectors (e.g., baculovirus); plant cell systems transformed with viral expression vectors (e.g., cauliflower mosaic virus, CaMV, or tobacco mosaic virus, TMV) or with bacterial expression vectors (e.g., Ti or pBR322 plasmids); or animal cell systems. (See, e.g., Sambrook, supra; Ausubel, supra; Van Heeke, G. and S.M. Schuster (1989) J. Biol. Chem. 264:5503-5509; Bitter, G.A. et al. (1987) Methods Enzymol. 153:516-544; Scorer, C.A. et al. (1994) Bio/Technology 12:181-184; Engelhard, E.K. et al. (1994) Proc. Natl. Acad. Sci. USA 91:3224-3227; Sandig, V. et al. (1996) Hum. Gene Ther. 7:1937-1945; Takamatsu, N. (1987) EMBO J. 6:307-311; Coruzzi, G. et al. (1984) EMBO J. 3:1671-1680; Broglie, R. et al. (1984) Science 224:838-843; Winter, J. et al. (1991) Results Probl. Cell Differ. 17:85-105; The McGraw Hill Yearbook of Science and Technology (1992) McGraw Hill, New York NY, pp. 191-196; Logan, J. and T. Shenk (1984) Proc. Natl. Acad. Sci. USA 81:3655-3659; and Harrington, J.J. et al. (1997) Nat. Genet. 15:345-355.) Expression vectors derived from retroviruses,

adenoviruses, or herpes or vaccinia viruses, or from various bacterial plasmids, may be used for delivery of nucleotide sequences to the targeted organ, tissue, or cell population. (See, e.g., Di Nicola, M. et al. (1998) Cancer Gen. Ther. 5(6):350-356; Yu, M. et al. (1993) Proc. Natl. Acad. Sci. USA 90(13):6340-6344; Buller, R.M. et al. (1985) Nature 317(6040):813-815; McGregor, D.P. et al. (1994) Mol. Immunol. 31(3):219-226; and Verma, I.M. and N. Somia (1997) Nature 389:239-242.) The invention is not limited by the host cell employed.

In bacterial systems, a number of cloning and expression vectors may be selected depending upon the use intended for polynucleotide sequences encoding CDIFF. For example, routine cloning, subcloning, and propagation of polynucleotide sequences encoding CDIFF can be achieved using a multifunctional E. coli vector such as PBLUESCRIPT (Stratagene, La Jolla CA) or PSPORT1 plasmid (Life Technologies). Ligation of sequences encoding CDIFF into the vector's multiple cloning site disrupts the *lacZ* gene, allowing a colorimetric screening procedure for identification of transformed bacteria containing recombinant molecules. In addition, these vectors may be useful for in vitro transcription, dideoxy sequencing, single strand rescue with helper phage, and creation of nested deletions in the cloned sequence. (See, e.g., Van Heeke, G. and S.M. Schuster (1989) J. Biol. Chem. 264:5503-5509.) When large quantities of CDIFF are needed, e.g. for the production of antibodies, vectors which direct high level expression of CDIFF may be used. For example, vectors containing the strong, inducible T5 or T7 bacteriophage promoter may be used.

Yeast expression systems may be used for production of CDIFF. A number of vectors containing constitutive or inducible promoters, such as alpha factor, alcohol oxidase, and PGH promoters, may be used in the yeast Saccharomyces cerevisiae or Pichia pastoris. In addition, such vectors direct either the secretion or intracellular retention of expressed proteins and enable integration of foreign sequences into the host genome for stable propagation. (See, e.g., Ausubel, 1995, supra; Bitter, supra; and Scorer, supra.)

Plant systems may also be used for expression of CDIFF. Transcription of sequences encoding CDIFF may be driven viral promoters, e.g., the 35S and 19S promoters of CaMV used alone or in combination with the omega leader sequence from TMV (Takamatsu, N. (1987) EMBO J. 6:307-311). Alternatively, plant promoters such as the small subunit of RUBISCO or heat shock promoters may be used. (See, e.g., Coruzzi, supra; Broglie, supra; and Winter, supra.) These constructs can be introduced into plant cells by direct DNA transformation or pathogen-mediated transfection. (See, e.g., The McGraw Hill Yearbook of Science and Technology (1992) McGraw Hill, New York NY, pp. 191-196.)

In mammalian cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, sequences encoding CDIFF may be ligated into an adenovirus transcription/translation complex consisting of the late promoter and tripartite leader

sequence. Insertion in a non-essential E1 or E3 region of the viral genome may be used to obtain infective virus which expresses CDIFF in host cells. (See, e.g., Logan, J. and T. Shenk (1984) Proc. Natl. Acad. Sci. USA 81:3655-3659.) In addition, transcription enhancers, such as the Rous sarcoma virus (RSV) enhancer, may be used to increase expression in mammalian host cells. SV40 or EBV-based vectors may also be used for high-level protein expression.

Human artificial chromosomes (HACs) may also be employed to deliver larger fragments of DNA than can be contained in and expressed from a plasmid. HACs of about 6 kb to 10 Mb are constructed and delivered via conventional delivery methods (liposomes, polycationic amino polymers, or vesicles) for therapeutic purposes. (See, e.g., Harrington, J.J. et al. (1997) Nat. Genet. 15:345-355.)

For long term production of recombinant proteins in mammalian systems, stable expression of CDIFF in cell lines is preferred. For example, sequences encoding CDIFF can be transformed into cell lines using expression vectors which may contain viral origins of replication and/or endogenous expression elements and a selectable marker gene on the same or on a separate vector. Following the introduction of the vector, cells may be allowed to grow for about 1 to 2 days in enriched media before being switched to selective media. The purpose of the selectable marker is to confer resistance to a selective agent, and its presence allows growth and recovery of cells which successfully express the introduced sequences. Resistant clones of stably transformed cells may be propagated using tissue culture techniques appropriate to the cell type.

Any number of selection systems may be used to recover transformed cell lines. These include, but are not limited to, the herpes simplex virus thymidine kinase and adenine phosphoribosyltransferase genes, for use in *tk*⁻ and *apr*⁻ cells, respectively. (See, e.g., Wigler, M. et al. (1977) Cell 11:223-232; Lowy, I. et al. (1980) Cell 22:817-823.) Also, antimetabolite, antibiotic, or herbicide resistance can be used as the basis for selection. For example, *dhfr* confers resistance to methotrexate; *neo* confers resistance to the aminoglycosides neomycin and G-418; and *als* and *pat* confer resistance to chlorsulfuron and phosphinotricin acetyltransferase, respectively. (See, e.g., Wigler, M. et al. (1980) Proc. Natl. Acad. Sci. USA 77:3567-3570; Colbere-Garapin, F. et al. (1981) J. Mol. Biol. 150:1-14.) Additional selectable genes have been described, e.g., *trpB* and *hisD*, which alter cellular requirements for metabolites. (See, e.g., Hartman, S.C. and R.C. Mulligan (1988) Proc. Natl. Acad. Sci. USA 85:8047-8051.) Visible markers, e.g., anthocyanins, green fluorescent proteins (GFP; Clontech), ß glucuronidase and its substrate ß-glucuronide, or luciferase and its substrate luciferin may be used. These markers can be used not only to identify transformants, but also to quantify the amount of transient or stable protein expression attributable to a specific vector system. (See, e.g., Rhodes, C.A. (1995) Methods Mol. Biol. 55:121-131.)

Although the presence/absence of marker gene expression suggests that the gene of interest is

also present, the presence and expression of the gene may need to be confirmed. For example, if the sequence encoding CDIFF is inserted within a marker gene sequence, transformed cells containing sequences encoding CDIFF can be identified by the absence of marker gene function. Alternatively, a marker gene can be placed in tandem with a sequence encoding CDIFF under the control of a single promoter. Expression of the marker gene in response to induction or selection usually indicates expression of the tandem gene as well.

In general, host cells that contain the nucleic acid sequence encoding CDIFF and that express CDIFF may be identified by a variety of procedures known to those of skill in the art. These procedures include, but are not limited to, DNA-DNA or DNA-RNA hybridizations, PCR amplification, and protein bioassay or immunoassay techniques which include membrane, solution, or chip based technologies for the detection and/or quantification of nucleic acid or protein sequences.

Immunological methods for detecting and measuring the expression of CDIFF using either specific polyclonal or monoclonal antibodies are known in the art. Examples of such techniques include enzyme-linked immunosorbent assays (ELISAs), radioimmunoassays (RIAs), and fluorescence activated cell sorting (FACS). A two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering epitopes on CDIFF is preferred, but a competitive binding assay may be employed. These and other assays are well known in the art. (See, e.g., Hampton, R. et al. (1990) Serological Methods, a Laboratory Manual, APS Press, St. Paul MN, Sect. IV; Coligan, J.E. et al. (1997) Current Protocols in Immunology, Greene Pub. Associates and Wiley-Interscience, New York NY; and Pound, J.D. (1998) Immunochemical Protocols, Humana Press, Totowa NJ.)

A wide variety of labels and conjugation techniques are known by those skilled in the art and may be used in various nucleic acid and amino acid assays. Means for producing labeled hybridization or PCR probes for detecting sequences related to polynucleotides encoding CDIFF include oligolabeling, nick translation, end-labeling, or PCR amplification using a labeled nucleotide. Alternatively, the sequences encoding CDIFF, or any fragments thereof, may be cloned into a vector for the production of an mRNA probe. Such vectors are known in the art, are commercially available, and may be used to synthesize RNA probes in vitro by addition of an appropriate RNA polymerase such as T7, T3, or SP6 and labeled nucleotides. These procedures may be conducted using a variety of commercially available kits, such as those provided by Amersham Pharmacia Biotech, Promega (Madison WI), and US Biochemical. Suitable reporter molecules or labels which may be used for ease of detection include radionuclides, enzymes, fluorescent, chemiluminescent, or chromogenic agents, as well as substrates, cofactors, inhibitors, magnetic particles, and the like.

Host cells transformed with nucleotide sequences encoding CDIFF may be cultured under conditions suitable for the expression and recovery of the protein from cell culture. The protein

produced by a transformed cell may be secreted or retained intracellularly depending on the sequence and/or the vector used. As will be understood by those of skill in the art, expression vectors containing polynucleotides which encode CDIFF may be designed to contain signal sequences which direct secretion of CDIFF through a prokaryotic or eukaryotic cell membrane.

In addition, a host cell strain may be chosen for its ability to modulate expression of the inserted sequences or to process the expressed protein in the desired fashion. Such modifications of the polypeptide include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation, and acylation. Post-translational processing which cleaves a "prepro" or "pro" form of the protein may also be used to specify protein targeting, folding, and/or activity. Different host cells which have specific cellular machinery and characteristic mechanisms for post-translational activities (e.g., CHO, HeLa, MDCK, HEK293, and WI38) are available from the American Type Culture Collection (ATCC, Manassas VA) and may be chosen to ensure the correct modification and processing of the foreign protein.

In another embodiment of the invention, natural, modified, or recombinant nucleic acid sequences encoding CDIFF may be ligated to a heterologous sequence resulting in translation of a fusion protein in any of the aforementioned host systems. For example, a chimeric CDIFF protein containing a heterologous moiety that can be recognized by a commercially available antibody may facilitate the screening of peptide libraries for inhibitors of CDIFF activity. Heterologous protein and peptide moieties may also facilitate purification of fusion proteins using commercially available affinity matrices. Such moieties include, but are not limited to, glutathione S-transferase (GST), maltose binding protein (MBP), thioredoxin (Trx), calmodulin binding peptide (CBP), 6-His, FLAG, *c-myc*, and hemagglutinin (HA). GST, MBP, Trx, CBP, and 6-His enable purification of their cognate fusion proteins on immobilized glutathione, maltose, phenylarsine oxide, calmodulin, and metal-chelate resins, respectively. FLAG, *c-myc*, and hemagglutinin (HA) enable immunoaffinity purification of fusion proteins using commercially available monoclonal and polyclonal antibodies that specifically recognize these epitope tags. A fusion protein may also be engineered to contain a proteolytic cleavage site located between the CDIFF encoding sequence and the heterologous protein sequence, so that CDIFF may be cleaved away from the heterologous moiety following purification. Methods for fusion protein expression and purification are discussed in Ausubel (1995, supra, ch. 10). A variety of commercially available kits may also be used to facilitate expression and purification of fusion proteins.

In a further embodiment of the invention, synthesis of radiolabeled CDIFF may be achieved in vitro using the TNT rabbit reticulocyte lysate or wheat germ extract system (Promega). These systems couple transcription and translation of protein-coding sequences operably associated with the T7, T3, or SP6 promoters. Translation takes place in the presence of a radiolabeled amino acid

precursor, for example, ^{35}S-methionine.

CDIFF of the present invention or fragments thereof may be used to screen for compounds that specifically bind to CDIFF. At least one and up to a plurality of test compounds may be screened for specific binding to CDIFF. Examples of test compounds include antibodies, oligonucleotides, proteins (e.g., receptors), or small molecules.

In one embodiment, the compound thus identified is closely related to the natural ligand of CDIFF, e.g., a ligand or fragment thereof, a natural substrate, a structural or functional mimetic, or a natural binding partner. (See, e.g., Coligan, J.E. et al. (1991) Current Protocols in Immunology 1(2): Chapter 5.) Similarly, the compound can be closely related to the natural receptor to which CDIFF binds, or to at least a fragment of the receptor, e.g., the ligand binding site. In either case, the compound can be rationally designed using known techniques. In one embodiment, screening for these compounds involves producing appropriate cells which express CDIFF, either as a secreted protein or on the cell membrane. Preferred cells include cells from mammals, yeast, Drosophila, or E. coli. Cells expressing CDIFF or cell membrane fractions which contain CDIFF are then contacted with a test compound and binding, stimulation, or inhibition of activity of either CDIFF or the compound is analyzed.

An assay may simply test binding of a test compound to the polypeptide, wherein binding is detected by a fluorophore, radioisotope, enzyme conjugate, or other detectable label. For example, the assay may comprise the steps of combining at least one test compound with CDIFF, either in solution or affixed to a solid support, and detecting the binding of CDIFF to the compound. Alternatively, the assay may detect or measure binding of a test compound in the presence of a labeled competitor. Additionally, the assay may be carried out using cell-free preparations, chemical libraries, or natural product mixtures, and the test compound(s) may be free in solution or affixed to a solid support.

CDIFF of the present invention or fragments thereof may be used to screen for compounds that modulate the activity of CDIFF. Such compounds may include agonists, antagonists, or partial or inverse agonists. In one embodiment, an assay is performed under conditions permissive for CDIFF activity, wherein CDIFF is combined with at least one test compound, and the activity of CDIFF in the presence of a test compound is compared with the activity of CDIFF in the absence of the test compound. A change in the activity of CDIFF in the presence of the test compound is indicative of a compound that modulates the activity of CDIFF. Alternatively, a test compound is combined with an in vitro or cell-free system comprising CDIFF under conditions suitable for CDIFF activity, and the assay is performed. In either of these assays, a test compound which modulates the activity of CDIFF may do so indirectly and need not come in direct contact with the test compound. At least one and up to a plurality of test compounds may be screened.

In another embodiment, polynucleotides encoding CDIFF or their mammalian homologs may be "knocked out" in an animal model system using homologous recombination in embryonic stem (ES) cells. Such techniques are well known in the art and are useful for the generation of animal models of human disease. (See, e.g., U.S. Patent No. 5,175,383 and U.S. Patent No. 5,767,337.) For example, mouse ES cells, such as the mouse 129/SvJ cell line, are derived from the early mouse embryo and grown in culture. The ES cells are transformed with a vector containing the gene of interest disrupted by a marker gene, e.g., the neomycin phosphotransferase gene (neo; Capecchi, M.R. (1989) Science 244:1288-1292). The vector integrates into the corresponding region of the host genome by homologous recombination. Alternatively, homologous recombination takes place using the Cre-loxP system to knockout a gene of interest in a tissue- or developmental stage-specific manner (Marth, J.D. (1996) Clin. Invest. 97:1999-2002; Wagner, K.U. et al. (1997) Nucleic Acids Res. 25:4323-4330). Transformed ES cells are identified and microinjected into mouse cell blastocysts such as those from the C57BL/6 mouse strain. The blastocysts are surgically transferred to pseudopregnant dams, and the resulting chimeric progeny are genotyped and bred to produce heterozygous or homozygous strains. Transgenic animals thus generated may be tested with potential therapeutic or toxic agents.

Polynucleotides encoding CDIFF may also be manipulated in vitro in ES cells derived from human blastocysts. Human ES cells have the potential to differentiate into at least eight separate cell lineages including endoderm, mesoderm, and ectodermal cell types. These cell lineages differentiate into, for example, neural cells, hematopoietic lineages, and cardiomyocytes (Thomson, J.A. et al. (1998) Science 282:1145-1147).

Polynucleotides encoding CDIFF can also be used to create "knockin" humanized animals (pigs) or transgenic animals (mice or rats) to model human disease. With knockin technology, a region of a polynucleotide encoding CDIFF is injected into animal ES cells, and the injected sequence integrates into the animal cell genome. Transformed cells are injected into blastulae, and the blastulae are implanted as described above. Transgenic progeny or inbred lines are studied and treated with potential pharmaceutical agents to obtain information on treatment of a human disease. Alternatively, a mammal inbred to overexpress CDIFF, e.g., by secreting CDIFF in its milk, may also serve as a convenient source of that protein (Janne, J. et al. (1998) Biotechnol. Annu. Rev. 4:55-74).

THERAPEUTICS

Chemical and structural similarity, e.g., in the context of sequences and motifs, exists between regions of CDIFF and proteins involved in cell differentiation. In addition, the expression of CDIFF is closely associated with cell proliferative disorders (including cancer) as well as reproductive and nervous tissue disorders. Therefore, CDIFF appears to play a role in cell proliferative, developmental, and neurological disorders. In the treatment of disorders associated

with increased CDIFF expression or activity, it is desirable to decrease the expression or activity of CDIFF. In the treatment of disorders associated with decreased CDIFF expression or activity, it is desirable to increase the expression or activity of CDIFF.

Therefore, in one embodiment, CDIFF or a fragment or derivative thereof may be administered to a subject to treat or prevent a disorder associated with decreased expression or activity of CDIFF. Examples of such disorders include, but are not limited to, a cell proliferative disorder such as actinic keratosis, arteriosclerosis, atherosclerosis, bursitis, cirrhosis, hepatitis, inflammatory disorders, mixed connective tissue disease (MCTD), myelofibrosis, paroxysmal nocturnal hemoglobinuria, polycythemia vera, psoriasis, primary thrombocythemia, and cancers including adenocarcinoma, leukemia, lymphoma, melanoma, myeloma, sarcoma, teratocarcinoma, and, in particular, cancers of the adrenal gland, bladder, bone, bone marrow, brain, breast, cervix, gall bladder, ganglia, gastrointestinal tract, heart, kidney, liver, lung, muscle, ovary, pancreas, parathyroid, penis, prostate, salivary glands, skin, spleen, testis, thymus, thyroid, and uterus; a developmental disorder such as renal tubular acidosis, anemia, Cushing's syndrome, achondroplastic dwarfism, Duchenne and Becker muscular dystrophy, epilepsy, gonadal dysgenesis, WAGR syndrome (Wilms' tumor, aniridia, genitourinary abnormalities, and mental retardation), Smith-Magenis syndrome, myelodysplastic syndrome, hereditary mucoepithelial dysplasia, hereditary keratodermas, hereditary neuropathies such as Charcot-Marie-Tooth disease and neurofibromatosis, hypothyroidism, hydrocephalus, seizure disorders such as Syndenham's chorea and cerebral palsy, spina bifida, anencephaly, craniorachischisis, congenital glaucoma, cataract, and sensorineural hearing loss; and a neurological disorder such as epilepsy, ischemic cerebrovascular disease, stroke, cerebral neoplasms, Alzheimer's disease, Pick's disease, Huntington's disease, dementia, Parkinson's disease and other extrapyramidal disorders, amyotrophic lateral sclerosis and other motor neuron disorders, progressive neural muscular atrophy, retinitis pigmentosa, hereditary ataxias, multiple sclerosis and other demyelinating diseases, bacterial and viral meningitis, brain abscess, subdural empyema, epidural abscess, suppurative intracranial thrombophlebitis, myelitis and radiculitis, viral central nervous system disease, prion diseases including kuru, Creutzfeldt-Jakob disease, and Gerstmann-Straussler-Scheinker syndrome, fatal familial insomnia, nutritional and metabolic diseases of the nervous system, neurofibromatosis, tuberous sclerosis, cerebelloretinal hemangioblastomatosis, encephalotrigeminal syndrome, mental retardation and other developmental disorders of the central nervous system, cerebral palsy, neuroskeletal disorders, autonomic nervous system disorders, cranial nerve disorders, spinal cord diseases, muscular dystrophy and other neuromuscular disorders, peripheral nervous system disorders, dermatomyositis and polymyositis; inherited, metabolic, endocrine, and toxic myopathies, myasthenia gravis, periodic paralysis; mental disorders including mood, anxiety, and schizophrenic disorders, seasonal affective disorder (SAD); akathesia, amnesia,

catatonia, diabetic neuropathy, tardive dyskinesia, dystonias, paranoid psychoses, postherpetic neuralgia, Tourette's disorder, progressive supranuclear palsy, corticobasal degeneration, and familial frontotemporal dementia.

In another embodiment, a vector capable of expressing CDIFF or a fragment or derivative thereof may be administered to a subject to treat or prevent a disorder associated with decreased expression or activity of CDIFF including, but not limited to, those described above.

In a further embodiment, a composition comprising a substantially purified CDIFF in conjunction with a suitable pharmaceutical carrier may be administered to a subject to treat or prevent a disorder associated with decreased expression or activity of CDIFF including, but not limited to, those provided above.

In still another embodiment, an agonist which modulates the activity of CDIFF may be administered to a subject to treat or prevent a disorder associated with decreased expression or activity of CDIFF including, but not limited to, those listed above.

In a further embodiment, an antagonist of CDIFF may be administered to a subject to treat or prevent a disorder associated with increased expression or activity of CDIFF. Examples of such disorders include, but are not limited to, those cell proliferative, developmental, and neurological disorders described above. In one aspect, an antibody which specifically binds CDIFF may be used directly as an antagonist or indirectly as a targeting or delivery mechanism for bringing a pharmaceutical agent to cells or tissues which express CDIFF.

In an additional embodiment, a vector expressing the complement of the polynucleotide encoding CDIFF may be administered to a subject to treat or prevent a disorder associated with increased expression or activity of CDIFF including, but not limited to, those described above.

In other embodiments, any of the proteins, antagonists, antibodies, agonists, complementary sequences, or vectors of the invention may be administered in combination with other appropriate therapeutic agents. Selection of the appropriate agents for use in combination therapy may be made by one of ordinary skill in the art, according to conventional pharmaceutical principles. The combination of therapeutic agents may act synergistically to effect the treatment or prevention of the various disorders described above. Using this approach, one may be able to achieve therapeutic efficacy with lower dosages of each agent, thus reducing the potential for adverse side effects.

An antagonist of CDIFF may be produced using methods which are generally known in the art. In particular, purified CDIFF may be used to produce antibodies or to screen libraries of pharmaceutical agents to identify those which specifically bind CDIFF. Antibodies to CDIFF may also be generated using methods that are well known in the art. Such antibodies may include, but are not limited to, polyclonal, monoclonal, chimeric, and single chain antibodies, Fab fragments, and fragments produced by a Fab expression library. Neutralizing antibodies (i.e., those which inhibit

dimer formation) are generally preferred for therapeutic use.

For the production of antibodies, various hosts including goats, rabbits, rats, mice, humans, and others may be immunized by injection with CDIFF or with any fragment or oligopeptide thereof which has immunogenic properties. Depending on the host species, various adjuvants may be used to increase immunological response. Such adjuvants include, but are not limited to, Freund's, mineral gels such as aluminum hydroxide, and surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, KLH, and dinitrophenol. Among adjuvants used in humans, BCG (bacilli Calmette-Guerin) and Corynebacterium parvum are especially preferable.

It is preferred that the oligopeptides, peptides, or fragments used to induce antibodies to CDIFF have an amino acid sequence consisting of at least about 5 amino acids, and generally will consist of at least about 10 amino acids. It is also preferable that these oligopeptides, peptides, or fragments are identical to a portion of the amino acid sequence of the natural protein. Short stretches of CDIFF amino acids may be fused with those of another protein, such as KLH, and antibodies to the chimeric molecule may be produced.

Monoclonal antibodies to CDIFF may be prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique, the human B-cell hybridoma technique, and the EBV-hybridoma technique. (See, e.g., Kohler, G. et al. (1975) Nature 256:495-497; Kozbor, D. et al. (1985) J. Immunol. Methods 81:31-42; Cote, R.J. et al. (1983) Proc. Natl. Acad. Sci. USA 80:2026-2030; and Cole, S.P. et al. (1984) Mol. Cell Biol. 62:109-120.)

In addition, techniques developed for the production of "chimeric antibodies," such as the splicing of mouse antibody genes to human antibody genes to obtain a molecule with appropriate antigen specificity and biological activity, can be used. (See, e.g., Morrison, S.L. et al. (1984) Proc. Natl. Acad. Sci. USA 81:6851-6855; Neuberger, M.S. et al. (1984) Nature 312:604-608; and Takeda, S. et al. (1985) Nature 314:452-454.) Alternatively, techniques described for the production of single chain antibodies may be adapted, using methods known in the art, to produce CDIFF-specific single chain antibodies. Antibodies with related specificity, but of distinct idiotypic composition, may be generated by chain shuffling from random combinatorial immunoglobulin libraries. (See, e.g., Burton, D.R. (1991) Proc. Natl. Acad. Sci. USA 88:10134-10137.)

Antibodies may also be produced by inducing in vivo production in the lymphocyte population or by screening immunoglobulin libraries or panels of highly specific binding reagents as disclosed in the literature. (See, e.g., Orlandi, R. et al. (1989) Proc. Natl. Acad. Sci. USA 86:3833-3837; Winter, G. et al. (1991) Nature 349:293-299.)

Antibody fragments which contain specific binding sites for CDIFF may also be generated. For example, such fragments include, but are not limited to, $F(ab')_2$ fragments produced by pepsin

digestion of the antibody molecule and Fab fragments generated by reducing the disulfide bridges of the F(ab')2 fragments. Alternatively, Fab expression libraries may be constructed to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity. (See, e.g., Huse, W.D. et al. (1989) Science 246:1275-1281.)

Various immunoassays may be used for screening to identify antibodies having the desired specificity. Numerous protocols for competitive binding or immunoradiometric assays using either polyclonal or monoclonal antibodies with established specificities are well known in the art. Such immunoassays typically involve the measurement of complex formation between CDIFF and its specific antibody. A two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering CDIFF epitopes is generally used, but a competitive binding assay may also be employed (Pound, supra).

Various methods such as Scatchard analysis in conjunction with radioimmunoassay techniques may be used to assess the affinity of antibodies for CDIFF. Affinity is expressed as an association constant, K_a, which is defined as the molar concentration of CDIFF-antibody complex divided by the molar concentrations of free antigen and free antibody under equilibrium conditions. The K_a determined for a preparation of polyclonal antibodies, which are heterogeneous in their affinities for multiple CDIFF epitopes, represents the average affinity, or avidity, of the antibodies for CDIFF. The K_a determined for a preparation of monoclonal antibodies, which are monospecific for a particular CDIFF epitope, represents a true measure of affinity. High-affinity antibody preparations with K_a ranging from about 10^9 to 10^{12} L/mole are preferred for use in immunoassays in which the CDIFF-antibody complex must withstand rigorous manipulations. Low-affinity antibody preparations with K_a ranging from about 10^6 to 10^7 L/mole are preferred for use in immunopurification and similar procedures which ultimately require dissociation of CDIFF, preferably in active form, from the antibody (Catty, D. (1988) Antibodies, Volume I: A Practical Approach, IRL Press, Washington DC; Liddell, J.E. and A. Cryer (1991) A Practical Guide to Monoclonal Antibodies, John Wiley & Sons, New York NY).

The titer and avidity of polyclonal antibody preparations may be further evaluated to determine the quality and suitability of such preparations for certain downstream applications. For example, a polyclonal antibody preparation containing at least 1-2 mg specific antibody/ml, preferably 5-10 mg specific antibody/ml, is generally employed in procedures requiring precipitation of CDIFF-antibody complexes. Procedures for evaluating antibody specificity, titer, and avidity, and guidelines for antibody quality and usage in various applications, are generally available. (See, e.g., Catty, supra, and Coligan et al., supra.)

In another embodiment of the invention, the polynucleotides encoding CDIFF, or any fragment or complement thereof, may be used for therapeutic purposes. In one aspect, modifications

of gene expression can be achieved by designing complementary sequences or antisense molecules (DNA, RNA, PNA, or modified oligonucleotides) to the coding or regulatory regions of the gene encoding CDIFF. Such technology is well known in the art, and antisense oligonucleotides or larger fragments can be designed from various locations along the coding or control regions of sequences encoding CDIFF. (See, e.g., Agrawal, S., ed. (1996) Antisense Therapeutics, Humana Press Inc., Totawa NJ.)

In therapeutic use, any gene delivery system suitable for introduction of the antisense sequences into appropriate target cells can be used. Antisense sequences can be delivered intracellularly in the form of an expression plasmid which, upon transcription, produces a sequence complementary to at least a portion of the cellular sequence encoding the target protein. (See, e.g., Slater, J.E. et al. (1998) J. Allergy Clin. Immunol. 102(3):469-475; and Scanlon, K.J. et al. (1995) 9(13):1288-1296.) Antisense sequences can also be introduced intracellularly through the use of viral vectors, such as retrovirus and adeno-associated virus vectors. (See, e.g., Miller, A.D. (1990) Blood 76:271; Ausubel, supra; Uckert, W. and W. Walther (1994) Pharmacol. Ther. 63(3):323-347.) Other gene delivery mechanisms include liposome-derived systems, artificial viral envelopes, and other systems known in the art. (See, e.g., Rossi, J.J. (1995) Br. Med. Bull. 51(1):217-225; Boado, R.J. et al. (1998) J. Pharm. Sci. 87(11):1308-1315; and Morris, M.C. et al. (1997) Nucleic Acids Res. 25(14):2730-2736.)

In another embodiment of the invention, polynucleotides encoding CDIFF may be used for somatic or germline gene therapy. Gene therapy may be performed to (i) correct a genetic deficiency (e.g., in the cases of severe combined immunodeficiency (SCID)-X1 disease characterized by X-linked inheritance (Cavazzana-Calvo, M. et al. (2000) Science 288:669-672), severe combined immunodeficiency syndrome associated with an inherited adenosine deaminase (ADA) deficiency (Blaese, R.M. et al. (1995) Science 270:475-480; Bordignon, C. et al. (1995) Science 270:470-475), cystic fibrosis (Zabner, J. et al. (1993) Cell 75:207-216; Crystal, R.G. et al. (1995) Hum. Gene Therapy 6:643-666; Crystal, R.G. et al. (1995) Hum. Gene Therapy 6:667-703), thalassamias, familial hypercholesterolemia, and hemophilia resulting from Factor VIII or Factor IX deficiencies (Crystal, R.G. (1995) Science 270:404-410; Verma, I.M. and N. Somia (1997) Nature 389:239-242)), (ii) express a conditionally lethal gene product (e.g., in the case of cancers which result from unregulated cell proliferation), or (iii) express a protein which affords protection against intracellular parasites (e.g., against human retroviruses, such as human immunodeficiency virus (HIV) (Baltimore, D. (1988) Nature 335:395-396; Poeschla, E. et al. (1996) Proc. Natl. Acad. Sci. USA. 93:11395-11399), hepatitis B or C virus (HBV, HCV); fungal parasites, such as Candida albicans and Paracoccidioides brasiliensis; and protozoan parasites such as Plasmodium falciparum and Trypanosoma cruzi). In the case where a genetic deficiency in CDIFF expression or regulation causes disease, the expression of

CDIFF from an appropriate population of transduced cells may alleviate the clinical manifestations caused by the genetic deficiency.

In a further embodiment of the invention, diseases or disorders caused by deficiencies in CDIFF are treated by constructing mammalian expression vectors encoding CDIFF and introducing these vectors by mechanical means into CDIFF-deficient cells. Mechanical transfer technologies for use with cells in vivo or ex vitro include (i) direct DNA microinjection into individual cells, (ii) ballistic gold particle delivery, (iii) liposome-mediated transfection, (iv) receptor-mediated gene transfer, and (v) the use of DNA transposons (Morgan, R.A. and W.F. Anderson (1993) Annu. Rev. Biochem. 62:191-217; Ivics, Z. (1997) Cell 91:501-510; Boulay, J-L. and H. Récipon (1998) Curr. Opin. Biotechnol. 9:445-450).

Expression vectors that may be effective for the expression of CDIFF include, but are not limited to, the PCDNA 3.1, EPITAG, PRCCMV2, PREP, PVAX vectors (Invitrogen, Carlsbad CA), PCMV-SCRIPT, PCMV-TAG, PEGSH/PERV (Stratagene, La Jolla CA), and PTET-OFF, PTET-ON, PTRE2, PTRE2-LUC, PTK-HYG (Clontech, Palo Alto CA). CDIFF may be expressed using (i) a constitutively active promoter, (e.g., from cytomegalovirus (CMV), Rous sarcoma virus (RSV), SV40 virus, thymidine kinase (TK), or β-actin genes), (ii) an inducible promoter (e.g., the tetracycline-regulated promoter (Gossen, M. and H. Bujard (1992) Proc. Natl. Acad. Sci. USA 89:5547-5551; Gossen, M. et al. (1995) Science 268:1766-1769; Rossi, F.M.V. and H.M. Blau (1998) Curr. Opin. Biotechnol. 9:451-456), commercially available in the T-REX plasmid (Invitrogen)); the ecdysone-inducible promoter (available in the plasmids PVGRXR and PIND; Invitrogen); the FK506/rapamycin inducible promoter; or the RU486/mifepristone inducible promoter (Rossi, F.M.V. and H.M. Blau, supra)), or (iii) a tissue-specific promoter or the native promoter of the endogenous gene encoding CDIFF from a normal individual.

Commercially available liposome transformation kits (e.g., the PERFECT LIPID TRANSFECTION KIT, available from Invitrogen) allow one with ordinary skill in the art to deliver polynucleotides to target cells in culture and require minimal effort to optimize experimental parameters. In the alternative, transformation is performed using the calcium phosphate method (Graham, F.L. and A.J. Eb (1973) Virology 52:456-467), or by electroporation (Neumann, E. et al. (1982) EMBO J. 1:841-845). The introduction of DNA to primary cells requires modification of these standardized mammalian transfection protocols.

In another embodiment of the invention, diseases or disorders caused by genetic defects with respect to CDIFF expression are treated by constructing a retrovirus vector consisting of (i) the polynucleotide encoding CDIFF under the control of an independent promoter or the retrovirus long terminal repeat (LTR) promoter, (ii) appropriate RNA packaging signals, and (iii) a Rev-responsive element (RRE) along with additional retrovirus *cis*-acting RNA sequences and coding sequences

required for efficient vector propagation. Retrovirus vectors (e.g., PFB and PFBNEO) are commercially available (Stratagene) and are based on published data (Riviere, I. et al. (1995) Proc. Natl. Acad. Sci. USA 92:6733-6737), incorporated by reference herein. The vector is propagated in an appropriate vector producing cell line (VPCL) that expresses an envelope gene with a tropism for receptors on the target cells or a promiscuous envelope protein such as VSVg (Armentano, D. et al. (1987) J. Virol. 61:1647-1650; Bender, M.A. et al. (1987) J. Virol. 61:1639-1646; Adam, M.A. and A.D. Miller (1988) J. Virol. 62:3802-3806; Dull, T. et al. (1998) J. Virol. 72:8463-8471; Zufferey, R. et al. (1998) J. Virol. 72:9873-9880). U.S. Patent Number 5,910,434 to Rigg ("Method for obtaining retrovirus packaging cell lines producing high transducing efficiency retroviral supernatant") discloses a method for obtaining retrovirus packaging cell lines and is hereby incorporated by reference. Propagation of retrovirus vectors, transduction of a population of cells (e.g., $CD4^+$ T-cells), and the return of transduced cells to a patient are procedures well known to persons skilled in the art of gene therapy and have been well documented (Ranga, U. et al. (1997) J. Virol. 71:7020-7029; Bauer, G. et al. (1997) Blood 89:2259-2267; Bonyhadi, M.L. (1997) J. Virol. 71:4707-4716; Ranga, U. et al. (1998) Proc. Natl. Acad. Sci. USA 95:1201-1206; Su, L. (1997) Blood 89:2283-2290).

In the alternative, an adenovirus-based gene therapy delivery system is used to deliver polynucleotides encoding CDIFF to cells which have one or more genetic abnormalities with respect to the expression of CDIFF. The construction and packaging of adenovirus-based vectors are well known to those with ordinary skill in the art. Replication defective adenovirus vectors have proven to be versatile for importing genes encoding immunoregulatory proteins into intact islets in the pancreas (Csete, M.E. et al. (1995) Transplantation 27:263-268). Potentially useful adenoviral vectors are described in U.S. Patent Number 5,707,618 to Armentano ("Adenovirus vectors for gene therapy"), hereby incorporated by reference. For adenoviral vectors, see also Antinozzi, P.A. et al. (1999) Annu. Rev. Nutr. 19:511-544; and Verma, I.M. and N. Somia (1997) Nature 18:389:239-242, both incorporated by reference herein.

In another alternative, a herpes-based, gene therapy delivery system is used to deliver polynucleotides encoding CDIFF to target cells which have one or more genetic abnormalities with respect to the expression of CDIFF. The use of herpes simplex virus (HSV)-based vectors may be especially valuable for introducing CDIFF to cells of the central nervous system, for which HSV has a tropism. The construction and packaging of herpes-based vectors are well known to those with ordinary skill in the art. A replication-competent herpes simplex virus (HSV) type 1-based vector has been used to deliver a reporter gene to the eyes of primates (Liu, X. et al. (1999) Exp. Eye Res.169:385-395). The construction of a HSV-1 virus vector has also been disclosed in detail in U.S. Patent Number 5,804,413 to DeLuca ("Herpes simplex virus strains for gene transfer"), which is

hereby incorporated by reference. U.S. Patent Number 5,804,413 teaches the use of recombinant HSV d92 which consists of a genome containing at least one exogenous gene to be transferred to a cell under the control of the appropriate promoter for purposes including human gene therapy. Also taught by this patent are the construction and use of recombinant HSV strains deleted for ICP4, ICP27 and ICP22. For HSV vectors, see also Goins, W.F. et al. (1999) J. Virol. 73:519-532 and Xu, H. et al. (1994) Dev. Biol. 163:152-161, hereby incorporated by reference. The manipulation of cloned herpesvirus sequences, the generation of recombinant virus following the transfection of multiple plasmids containing different segments of the large herpesvirus genomes, the growth and propagation of herpesvirus, and the infection of cells with herpesvirus are techniques well known to those of ordinary skill in the art.

In another alternative, an alphavirus (positive, single-stranded RNA virus) vector is used to deliver polynucleotides encoding CDIFF to target cells. The biology of the prototypic alphavirus, Semliki Forest Virus (SFV), has been studied extensively and gene transfer vectors have been based on the SFV genome (Garoff, H. and K.-J. Li (1998) Curr. Opin. Biotechnol. 9:464-469). During alphavirus RNA replication, a subgenomic RNA is generated that normally encodes the viral capsid proteins. This subgenomic RNA replicates to higher levels than the full-length genomic RNA, resulting in the overproduction of capsid proteins relative to the viral proteins with enzymatic activity (e.g., protease and polymerase). Similarly, inserting the coding sequence for CDIFF into the alphavirus genome in place of the capsid-coding region results in the production of a large number of CDIFF-coding RNAs and the synthesis of high levels of CDIFF in vector transduced cells. While alphavirus infection is typically associated with cell lysis within a few days, the ability to establish a persistent infection in hamster normal kidney cells (BHK-21) with a variant of Sindbis virus (SIN) indicates that the lytic replication of alphaviruses can be altered to suit the needs of the gene therapy application (Dryga, S.A. et al. (1997) Virology 228:74-83). The wide host range of alphaviruses will allow the introduction of CDIFF into a variety of cell types. The specific transduction of a subset of cells in a population may require the sorting of cells prior to transduction. The methods of manipulating infectious cDNA clones of alphaviruses, performing alphavirus cDNA and RNA transfections, and performing alphavirus infections, are well known to those with ordinary skill in the art.

Oligonucleotides derived from the transcription initiation site, e.g., between about positions -10 and +10 from the start site, may also be employed to inhibit gene expression. Similarly, inhibition can be achieved using triple helix base-pairing methodology. Triple helix pairing is useful because it causes inhibition of the ability of the double helix to open sufficiently for the binding of polymerases, transcription factors, or regulatory molecules. Recent therapeutic advances using triplex DNA have been described in the literature. (See, e.g., Gee, J.E. et al. (1994) in Huber, B.E.

and B.I. Carr, Molecular and Immunologic Approaches, Futura Publishing, Mt. Kisco NY, pp. 163-177.) A complementary sequence or antisense molecule may also be designed to block translation of mRNA by preventing the transcript from binding to ribosomes.

Ribozymes, enzymatic RNA molecules, may also be used to catalyze the specific cleavage of RNA. The mechanism of ribozyme action involves sequence-specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. For example, engineered hammerhead motif ribozyme molecules may specifically and efficiently catalyze endonucleolytic cleavage of sequences encoding CDIFF.

Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites, including the following sequences: GUA, GUU, and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides, corresponding to the region of the target gene containing the cleavage site, may be evaluated for secondary structural features which may render the oligonucleotide inoperable. The suitability of candidate targets may also be evaluated by testing accessibility to hybridization with complementary oligonucleotides using ribonuclease protection assays.

Complementary ribonucleic acid molecules and ribozymes of the invention may be prepared by any method known in the art for the synthesis of nucleic acid molecules. These include techniques for chemically synthesizing oligonucleotides such as solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by in vitro and in vivo transcription of DNA sequences encoding CDIFF. Such DNA sequences may be incorporated into a wide variety of vectors with suitable RNA polymerase promoters such as T7 or SP6. Alternatively, these cDNA constructs that synthesize complementary RNA, constitutively or inducibly, can be introduced into cell lines, cells, or tissues.

RNA molecules may be modified to increase intracellular stability and half-life. Possible modifications include, but are not limited to, the addition of flanking sequences at the 5' and/or 3' ends of the molecule, or the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages within the backbone of the molecule. This concept is inherent in the production of PNAs and can be extended in all of these molecules by the inclusion of nontraditional bases such as inosine, queosine, and wybutosine, as well as acetyl-, methyl-, thio-, and similarly modified forms of adenine, cytidine, guanine, thymine, and uridine which are not as easily recognized by endogenous endonucleases.

An additional embodiment of the invention encompasses a method for screening for a compound which is effective in altering expression of a polynucleotide encoding CDIFF. Compounds which may be effective in altering expression of a specific polynucleotide may include, but are not limited to, oligonucleotides, antisense oligonucleotides, triple helix-forming

oligonucleotides, transcription factors and other polypeptide transcriptional regulators, and non-macromolecular chemical entities which are capable of interacting with specific polynucleotide sequences. Effective compounds may alter polynucleotide expression by acting as either inhibitors or promoters of polynucleotide expression. Thus, in the treatment of disorders associated with increased CDIFF expression or activity, a compound which specifically inhibits expression of the polynucleotide encoding CDIFF may be therapeutically useful, and in the treament of disorders associated with decreased CDIFF expression or activity, a compound which specifically promotes expression of the polynucleotide encoding CDIFF may be therapeutically useful.

At least one, and up to a plurality, of test compounds may be screened for effectiveness in altering expression of a specific polynucleotide. A test compound may be obtained by any method commonly known in the art, including chemical modification of a compound known to be effective in altering polynucleotide expression; selection from an existing, commercially-available or proprietary library of naturally-occurring or non-natural chemical compounds; rational design of a compound based on chemical and/or structural properties of the target polynucleotide; and selection from a library of chemical compounds created combinatorially or randomly. A sample comprising a polynucleotide encoding CDIFF is exposed to at least one test compound thus obtained. The sample may comprise, for example, an intact or permeabilized cell, or an in vitro cell-free or reconstituted biochemical system. Alterations in the expression of a polynucleotide encoding CDIFF are assayed by any method commonly known in the art. Typically, the expression of a specific nucleotide is detected by hybridization with a probe having a nucleotide sequence complementary to the sequence of the polynucleotide encoding CDIFF. The amount of hybridization may be quantified, thus forming the basis for a comparison of the expression of the polynucleotide both with and without exposure to one or more test compounds. Detection of a change in the expression of a polynucleotide exposed to a test compound indicates that the test compound is effective in altering the expression of the polynucleotide. A screen for a compound effective in altering expression of a specific polynucleotide can be carried out, for example, using a Schizosaccharomyces pombe gene expression system (Atkins, D. et al. (1999) U.S. Patent No. 5,932,435; Arndt, G.M. et al. (2000) Nucleic Acids Res. 28:E15) or a human cell line such as HeLa cell (Clarke, M.L. et al. (2000) Biochem. Biophys. Res. Commun. 268:8-13). A particular embodiment of the present invention involves screening a combinatorial library of oligonucleotides (such as deoxyribonucleotides, ribonucleotides, peptide nucleic acids, and modified oligonucleotides) for antisense activity against a specific polynucleotide sequence (Bruice, T.W. et al. (1997) U.S. Patent No. 5,686,242; Bruice, T.W. et al. (2000) U.S. Patent No. 6,022,691).

Many methods for introducing vectors into cells or tissues are available and equally suitable for use in vivo, in vitro, and ex vivo. For ex vivo therapy, vectors may be introduced into stem cells

taken from the patient and clonally propagated for autologous transplant back into that same patient. Delivery by transfection, by liposome injections, or by polycationic amino polymers may be achieved using methods which are well known in the art. (See, e.g., Goldman, C.K. et al. (1997) Nat. Biotechnol. 15:462-466.)

Any of the therapeutic methods described above may be applied to any subject in need of such therapy, including, for example, mammals such as humans, dogs, cats, cows, horses, rabbits, and monkeys.

An additional embodiment of the invention relates to the administration of a composition which generally comprises an active ingredient formulated with a pharmaceutically acceptable excipient. Excipients may include, for example, sugars, starches, celluloses, gums, and proteins. Various formulations are commonly known and are thoroughly discussed in the latest edition of Remington's Pharmaceutical Sciences (Maack Publishing, Easton PA). Such compositions may consist of CDIFF, antibodies to CDIFF, and mimetics, agonists, antagonists, or inhibitors of CDIFF.

The compositions utilized in this invention may be administered by any number of routes including, but not limited to, oral, intravenous, intramuscular, intra-arterial, intramedullary, intrathecal, intraventricular, pulmonary, transdermal, subcutaneous, intraperitoneal, intranasal, enteral, topical, sublingual, or rectal means.

Compositions for pulmonary administration may be prepared in liquid or dry powder form. These compositions are generally aerosolized immediately prior to inhalation by the patient. In the case of small molecules (e.g. traditional low molecular weight organic drugs), aerosol delivery of fast-acting formulations is well-known in the art. In the case of macromolecules (e.g. larger peptides and proteins), recent developments in the field of pulmonary delivery via the alveolar region of the lung have enabled the practical delivery of drugs such as insulin to blood circulation (see, e.g., Patton, J.S. et al., U.S. Patent No. 5,997,848). Pulmonary delivery has the advantage of administration without needle injection, and obviates the need for potentially toxic penetration enhancers.

Compositions suitable for use in the invention include compositions wherein the active ingredients are contained in an effective amount to achieve the intended purpose. The determination of an effective dose is well within the capability of those skilled in the art.

Specialized forms of compositions may be prepared for direct intracellular delivery of macromolecules comprising CDIFF or fragments thereof. For example, liposome preparations containing a cell-impermeable macromolecule may promote cell fusion and intracellular delivery of the macromolecule. Alternatively, CDIFF or a fragment thereof may be joined to a short cationic N-terminal portion from the HIV Tat-1 protein. Fusion proteins thus generated have been found to transduce into the cells of all tissues, including the brain, in a mouse model system (Schwarze, S.R. et al. (1999) Science 285:1569-1572).

For any compound, the therapeutically effective dose can be estimated initially either in cell culture assays, e.g., of neoplastic cells, or in animal models such as mice, rats, rabbits, dogs, monkeys, or pigs. An animal model may also be used to determine the appropriate concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in humans.

A therapeutically effective dose refers to that amount of active ingredient, for example CDIFF or fragments thereof, antibodies of CDIFF, and agonists, antagonists or inhibitors of CDIFF, which ameliorates the symptoms or condition. Therapeutic efficacy and toxicity may be determined by standard pharmaceutical procedures in cell cultures or with experimental animals, such as by calculating the ED_{50} (the dose therapeutically effective in 50% of the population) or LD_{50} (the dose lethal to 50% of the population) statistics. The dose ratio of toxic to therapeutic effects is the therapeutic index, which can be expressed as the LD_{50}/ED_{50} ratio. Compositions which exhibit large therapeutic indices are preferred. The data obtained from cell culture assays and animal studies are used to formulate a range of dosage for human use. The dosage contained in such compositions is preferably within a range of circulating concentrations that includes the ED_{50} with little or no toxicity. The dosage varies within this range depending upon the dosage form employed, the sensitivity of the patient, and the route of administration.

The exact dosage will be determined by the practitioner, in light of factors related to the subject requiring treatment. Dosage and administration are adjusted to provide sufficient levels of the active moiety or to maintain the desired effect. Factors which may be taken into account include the severity of the disease state, the general health of the subject, the age, weight, and gender of the subject, time and frequency of administration, drug combination(s), reaction sensitivities, and response to therapy. Long-acting compositions may be administered every 3 to 4 days, every week, or biweekly depending on the half-life and clearance rate of the particular formulation.

Normal dosage amounts may vary from about 0.1 μg to 100,000 μg, up to a total dose of about 1 gram, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature and generally available to practitioners in the art. Those skilled in the art will employ different formulations for nucleotides than for proteins or their inhibitors. Similarly, delivery of polynucleotides or polypeptides will be specific to particular cells, conditions, locations, etc.

DIAGNOSTICS

In another embodiment, antibodies which specifically bind CDIFF may be used for the diagnosis of disorders characterized by expression of CDIFF, or in assays to monitor patients being treated with CDIFF or agonists, antagonists, or inhibitors of CDIFF. Antibodies useful for diagnostic purposes may be prepared in the same manner as described above for therapeutics. Diagnostic assays

for CDIFF include methods which utilize the antibody and a label to detect CDIFF in human body fluids or in extracts of cells or tissues. The antibodies may be used with or without modification, and may be labeled by covalent or non-covalent attachment of a reporter molecule. A wide variety of reporter molecules, several of which are described above, are known in the art and may be used.

A variety of protocols for measuring CDIFF, including ELISAs, RIAs, and FACS, are known in the art and provide a basis for diagnosing altered or abnormal levels of CDIFF expression. Normal or standard values for CDIFF expression are established by combining body fluids or cell extracts taken from normal mammalian subjects, for example, human subjects, with antibody to CDIFF under conditions suitable for complex formation. The amount of standard complex formation may be quantitated by various methods, such as photometric means. Quantities of CDIFF expressed in subject, control, and disease samples from biopsied tissues are compared with the standard values. Deviation between standard and subject values establishes the parameters for diagnosing disease.

In another embodiment of the invention, the polynucleotides encoding CDIFF may be used for diagnostic purposes. The polynucleotides which may be used include oligonucleotide sequences, complementary RNA and DNA molecules, and PNAs. The polynucleotides may be used to detect and quantify gene expression in biopsied tissues in which expression of CDIFF may be correlated with disease. The diagnostic assay may be used to determine absence, presence, and excess expression of CDIFF, and to monitor regulation of CDIFF levels during therapeutic intervention.

In one aspect, hybridization with PCR probes which are capable of detecting polynucleotide sequences, including genomic sequences, encoding CDIFF or closely related molecules may be used to identify nucleic acid sequences which encode CDIFF. The specificity of the probe, whether it is made from a highly specific region, e.g., the 5' regulatory region, or from a less specific region, e.g., a conserved motif, and the stringency of the hybridization or amplification will determine whether the probe identifies only naturally occurring sequences encoding CDIFF, allelic variants, or related sequences.

Probes may also be used for the detection of related sequences, and may have at least 50% sequence identity to any of the CDIFF encoding sequences. The hybridization probes of the subject invention may be DNA or RNA and may be derived from the sequence of SEQ ID NO:29-56 or from genomic sequences including promoters, enhancers, and introns of the CDIFF gene.

Means for producing specific hybridization probes for DNAs encoding CDIFF include the cloning of polynucleotide sequences encoding CDIFF or CDIFF derivatives into vectors for the production of mRNA probes. Such vectors are known in the art, are commercially available, and may be used to synthesize RNA probes in vitro by means of the addition of the appropriate RNA polymerases and the appropriate labeled nucleotides. Hybridization probes may be labeled by a variety of reporter groups, for example, by radionuclides such as ^{32}P or ^{35}S, or by enzymatic labels,

such as alkaline phosphatase coupled to the probe via avidin/biotin coupling systems, and the like.

Polynucleotide sequences encoding CDIFF may be used for the diagnosis of disorders associated with expression of CDIFF. Examples of such disorders include, but are not limited to, a cell proliferative disorder such as actinic keratosis, arteriosclerosis, atherosclerosis, bursitis, cirrhosis, hepatitis, inflammatory disorders, mixed connective tissue disease (MCTD), myelofibrosis, paroxysmal nocturnal hemoglobinuria, polycythemia vera, psoriasis, primary thrombocythemia, and cancers including adenocarcinoma, leukemia, lymphoma, melanoma, myeloma, sarcoma, teratocarcinoma, and, in particular, cancers of the adrenal gland, bladder, bone, bone marrow, brain, breast, cervix, gall bladder, ganglia, gastrointestinal tract, heart, kidney, liver, lung, muscle, ovary, pancreas, parathyroid, penis, prostate, salivary glands, skin, spleen, testis, thymus, thyroid, and uterus; a developmental disorder such as renal tubular acidosis, anemia, Cushing's syndrome, achondroplastic dwarfism, Duchenne and Becker muscular dystrophy, epilepsy, gonadal dysgenesis, WAGR syndrome (Wilms' tumor, aniridia, genitourinary abnormalities, and mental retardation), Smith-Magenis syndrome, myelodysplastic syndrome, hereditary mucoepithelial dysplasia, hereditary keratodermas, hereditary neuropathies such as Charcot-Marie-Tooth disease and neurofibromatosis, hypothyroidism, hydrocephalus, seizure disorders such as Syndenham's chorea and cerebral palsy, spina bifida, anencephaly, craniorachischisis, congenital glaucoma, cataract, and sensorineural hearing loss; and a neurological disorder such as epilepsy, ischemic cerebrovascular disease, stroke, cerebral neoplasms, Alzheimer's disease, Pick's disease, Huntington's disease, dementia, Parkinson's disease and other extrapyramidal disorders, amyotrophic lateral sclerosis and other motor neuron disorders, progressive neural muscular atrophy, retinitis pigmentosa, hereditary ataxias, multiple sclerosis and other demyelinating diseases, bacterial and viral meningitis, brain abscess, subdural empyema, epidural abscess, suppurative intracranial thrombophlebitis, myelitis and radiculitis, viral central nervous system disease, prion diseases including kuru, Creutzfeldt-Jakob disease, and Gerstmann-Straussler-Scheinker syndrome, fatal familial insomnia, nutritional and metabolic diseases of the nervous system, neurofibromatosis, tuberous sclerosis, cerebelloretinal hemangioblastomatosis, encephalotrigeminal syndrome, mental retardation and other developmental disorders of the central nervous system, cerebral palsy, neuroskeletal disorders, autonomic nervous system disorders, cranial nerve disorders, spinal cord diseases, muscular dystrophy and other neuromuscular disorders, peripheral nervous system disorders, dermatomyositis and polymyositis; inherited, metabolic, endocrine, and toxic myopathies, myasthenia gravis, periodic paralysis; mental disorders including mood, anxiety, and schizophrenic disorders, seasonal affective disorder (SAD); akathesia, amnesia, catatonia, diabetic neuropathy, tardive dyskinesia, dystonias, paranoid psychoses, postherpetic neuralgia, Tourette's disorder, progressive supranuclear palsy, corticobasal degeneration, and familial frontotemporal dementia. The polynucleotide sequences encoding CDIFF may be used in Southern or

northern analysis, dot blot, or other membrane-based technologies; in PCR technologies; in dipstick, pin, and multiformat ELISA-like assays; and in microarrays utilizing fluids or tissues from patients to detect altered CDIFF expression. Such qualitative or quantitative methods are well known in the art.

In a particular aspect, the nucleotide sequences encoding CDIFF may be useful in assays that detect the presence of associated disorders, particularly those mentioned above. The nucleotide sequences encoding CDIFF may be labeled by standard methods and added to a fluid or tissue sample from a patient under conditions suitable for the formation of hybridization complexes. After a suitable incubation period, the sample is washed and the signal is quantified and compared with a standard value. If the amount of signal in the patient sample is significantly altered in comparison to a control sample then the presence of altered levels of nucleotide sequences encoding CDIFF in the sample indicates the presence of the associated disorder. Such assays may also be used to evaluate the efficacy of a particular therapeutic treatment regimen in animal studies, in clinical trials, or to monitor the treatment of an individual patient.

In order to provide a basis for the diagnosis of a disorder associated with expression of CDIFF, a normal or standard profile for expression is established. This may be accomplished by combining body fluids or cell extracts taken from normal subjects, either animal or human, with a sequence, or a fragment thereof, encoding CDIFF, under conditions suitable for hybridization or amplification. Standard hybridization may be quantified by comparing the values obtained from normal subjects with values from an experiment in which a known amount of a substantially purified polynucleotide is used. Standard values obtained in this manner may be compared with values obtained from samples from patients who are symptomatic for a disorder. Deviation from standard values is used to establish the presence of a disorder.

Once the presence of a disorder is established and a treatment protocol is initiated, hybridization assays may be repeated on a regular basis to determine if the level of expression in the patient begins to approximate that which is observed in the normal subject. The results obtained from successive assays may be used to show the efficacy of treatment over a period ranging from several days to months.

With respect to cancer, the presence of an abnormal amount of transcript (either under- or overexpressed) in biopsied tissue from an individual may indicate a predisposition for the development of the disease, or may provide a means for detecting the disease prior to the appearance of actual clinical symptoms. A more definitive diagnosis of this type may allow health professionals to employ preventative measures or aggressive treatment earlier thereby preventing the development or further progression of the cancer.

Additional diagnostic uses for oligonucleotides designed from the sequences encoding CDIFF may involve the use of PCR. These oligomers may be chemically synthesized, generated

enzymatically, or produced in vitro. Oligomers will preferably contain a fragment of a polynucleotide encoding CDIFF, or a fragment of a polynucleotide complementary to the polynucleotide encoding CDIFF, and will be employed under optimized conditions for identification of a specific gene or condition. Oligomers may also be employed under less stringent conditions for detection or quantification of closely related DNA or RNA sequences.

In a particular aspect, oligonucleotide primers derived from the polynucleotide sequences encoding CDIFF may be used to detect single nucleotide polymorphisms (SNPs). SNPs are substitutions, insertions and deletions that are a frequent cause of inherited or acquired genetic disease in humans. Methods of SNP detection include, but are not limited to, single-stranded conformation polymorphism (SSCP) and fluorescent SSCP (fSSCP) methods. In SSCP, oligonucleotide primers derived from the polynucleotide sequences encoding CDIFF are used to amplify DNA using the polymerase chain reaction (PCR). The DNA may be derived, for example, from diseased or normal tissue, biopsy samples, bodily fluids, and the like. SNPs in the DNA cause differences in the secondary and tertiary structures of PCR products in single-stranded form, and these differences are detectable using gel electrophoresis in non-denaturing gels. In fSCCP, the oligonucleotide primers are fluorescently labeled, which allows detection of the amplimers in high-throughput equipment such as DNA sequencing machines. Additionally, sequence database analysis methods, termed in silico SNP (isSNP), are capable of identifying polymorphisms by comparing the sequence of individual overlapping DNA fragments which assemble into a common consensus sequence. These computer-based methods filter out sequence variations due to laboratory preparation of DNA and sequencing errors using statistical models and automated analyses of DNA sequence chromatograms. In the alternative, SNPs may be detected and characterized by mass spectrometry using, for example, the high throughput MASSARRAY system (Sequenom, Inc., San Diego CA).

Methods which may also be used to quantify the expression of CDIFF include radiolabeling or biotinylating nucleotides, coamplification of a control nucleic acid, and interpolating results from standard curves. (See, e.g., Melby, P.C. et al. (1993) J. Immunol. Methods 159:235-244; Duplaa, C. et al. (1993) Anal. Biochem. 212:229-236.) The speed of quantitation of multiple samples may be accelerated by running the assay in a high-throughput format where the oligomer or polynucleotide of interest is presented in various dilutions and a spectrophotometric or colorimetric response gives rapid quantitation.

In further embodiments, oligonucleotides or longer fragments derived from any of the polynucleotide sequences described herein may be used as elements on a microarray. The microarray can be used in transcript imaging techniques which monitor the relative expression levels of large numbers of genes simultaneously as described in Seilhamer, J.J. et al., "Comparative Gene Transcript Analysis," U.S. Patent No. 5,840,484, incorporated herein by reference. The microarray may also be

used to identify genetic variants, mutations, and polymorphisms. This information may be used to determine gene function, to understand the genetic basis of a disorder, to diagnose a disorder, to monitor progression/regression of disease as a function of gene expression, and to develop and monitor the activities of therapeutic agents in the treatment of disease. In particular, this information may be used to develop a pharmacogenomic profile of a patient in order to select the most appropriate and effective treatment regimen for that patient. For example, therapeutic agents which are highly effective and display the fewest side effects may be selected for a patient based on his/her pharmacogenomic profile.

In another embodiment, antibodies specific for CDIFF, or CDIFF or fragments thereof may be used as elements on a microarray. The microarray may be used to monitor or measure protein-protein interactions, drug-target interactions, and gene expression profiles, as described above.

A particular embodiment relates to the use of the polynucleotides of the present invention to generate a transcript image of a tissue or cell type. A transcript image represents the global pattern of gene expression by a particular tissue or cell type. Global gene expression patterns are analyzed by quantifying the number of expressed genes and their relative abundance under given conditions and at a given time. (See Seilhamer et al., "Comparative Gene Transcript Analysis," U.S. Patent Number 5,840,484, expressly incorporated by reference herein.) Thus a transcript image may be generated by hybridizing the polynucleotides of the present invention or their complements to the totality of transcripts or reverse transcripts of a particular tissue or cell type. In one embodiment, the hybridization takes place in high-throughput format, wherein the polynucleotides of the present invention or their complements comprise a subset of a plurality of elements on a microarray. The resultant transcript image would provide a profile of gene activity.

Transcript images may be generated using transcripts isolated from tissues, cell lines, biopsies, or other biological samples. The transcript image may thus reflect gene expression in vivo, as in the case of a tissue or biopsy sample, or in vitro, as in the case of a cell line.

Transcript images which profile the expression of the polynucleotides of the present invention may also be used in conjunction with in vitro model systems and preclinical evaluation of pharmaceuticals, as well as toxicological testing of industrial and naturally-occurring environmental compounds. All compounds induce characteristic gene expression patterns, frequently termed molecular fingerprints or toxicant signatures, which are indicative of mechanisms of action and toxicity (Nuwaysir, E.F. et al. (1999) Mol. Carcinog. 24:153-159; Steiner, S. and N.L. Anderson (2000) Toxicol. Lett. 112-113:467-471, expressly incorporated by reference herein). If a test compound has a signature similar to that of a compound with known toxicity, it is likely to share those toxic properties. These fingerprints or signatures are most useful and refined when they contain expression information from a large number of genes and gene families. Ideally, a genome-wide

measurement of expression provides the highest quality signature. Even genes whose expression is not altered by any tested compounds are important as well, as the levels of expression of these genes are used to normalize the rest of the expression data. The normalization procedure is useful for comparison of expression data after treatment with different compounds. While the assignment of gene function to elements of a toxicant signature aids in interpretation of toxicity mechanisms, knowledge of gene function is not necessary for the statistical matching of signatures which leads to prediction of toxicity. (See, for example, Press Release 00-02 from the National Institute of Environmental Health Sciences, released February 29, 2000, available at http://www.niehs.nih.gov/oc/news/toxchip.htm.) Therefore, it is important and desirable in toxicological screening using toxicant signatures to include all expressed gene sequences.

In one embodiment, the toxicity of a test compound is assessed by treating a biological sample containing nucleic acids with the test compound. Nucleic acids that are expressed in the treated biological sample are hybridized with one or more probes specific to the polynucleotides of the present invention, so that transcript levels corresponding to the polynucleotides of the present invention may be quantified. The transcript levels in the treated biological sample are compared with levels in an untreated biological sample. Differences in the transcript levels between the two samples are indicative of a toxic response caused by the test compound in the treated sample.

Another particular embodiment relates to the use of the polypeptide sequences of the present invention to analyze the proteome of a tissue or cell type. The term proteome refers to the global pattern of protein expression in a particular tissue or cell type. Each protein component of a proteome can be subjected individually to further analysis. Proteome expression patterns, or profiles, are analyzed by quantifying the number of expressed proteins and their relative abundance under given conditions and at a given time. A profile of a cell's proteome may thus be generated by separating and analyzing the polypeptides of a particular tissue or cell type. In one embodiment, the separation is achieved using two-dimensional gel electrophoresis, in which proteins from a sample are separated by isoelectric focusing in the first dimension, and then according to molecular weight by sodium dodecyl sulfate slab gel electrophoresis in the second dimension (Steiner and Anderson, supra). The proteins are visualized in the gel as discrete and uniquely positioned spots, typically by staining the gel with an agent such as Coomassie Blue or silver or fluorescent stains. The optical density of each protein spot is generally proportional to the level of the protein in the sample. The optical densities of equivalently positioned protein spots from different samples, for example, from biological samples either treated or untreated with a test compound or therapeutic agent, are compared to identify any changes in protein spot density related to the treatment. The proteins in the spots are partially sequenced using, for example, standard methods employing chemical or enzymatic cleavage followed by mass spectrometry. The identity of the protein in a spot may be determined by

comparing its partial sequence, preferably of at least 5 contiguous amino acid residues, to the polypeptide sequences of the present invention. In some cases, further sequence data may be obtained for definitive protein identification.

A proteomic profile may also be generated using antibodies specific for CDIFF to quantify the levels of CDIFF expression. In one embodiment, the antibodies are used as elements on a microarray, and protein expression levels are quantified by exposing the microarray to the sample and detecting the levels of protein bound to each array element (Lueking, A. et al. (1999) Anal. Biochem. 270:103-111; Mendoze, L.G. et al. (1999) Biotechniques 27:778-788). Detection may be performed by a variety of methods known in the art, for example, by reacting the proteins in the sample with a thiol- or amino-reactive fluorescent compound and detecting the amount of fluorescence bound at each array element.

Toxicant signatures at the proteome level are also useful for toxicological screening, and should be analyzed in parallel with toxicant signatures at the transcript level. There is a poor correlation between transcript and protein abundances for some proteins in some tissues (Anderson, N.L. and J. Seilhamer (1997) Electrophoresis 18:533-537), so proteome toxicant signatures may be useful in the analysis of compounds which do not significantly affect the transcript image, but which alter the proteomic profile. In addition, the analysis of transcripts in body fluids is difficult, due to rapid degradation of mRNA, so proteomic profiling may be more reliable and informative in such cases.

In another embodiment, the toxicity of a test compound is assessed by treating a biological sample containing proteins with the test compound. Proteins that are expressed in the treated biological sample are separated so that the amount of each protein can be quantified. The amount of each protein is compared to the amount of the corresponding protein in an untreated biological sample. A difference in the amount of protein between the two samples is indicative of a toxic response to the test compound in the treated sample. Individual proteins are identified by sequencing the amino acid residues of the individual proteins and comparing these partial sequences to the polypeptides of the present invention.

In another embodiment, the toxicity of a test compound is assessed by treating a biological sample containing proteins with the test compound. Proteins from the biological sample are incubated with antibodies specific to the polypeptides of the present invention. The amount of protein recognized by the antibodies is quantified. The amount of protein in the treated biological sample is compared with the amount in an untreated biological sample. A difference in the amount of protein between the two samples is indicative of a toxic response to the test compound in the treated sample.

Microarrays may be prepared, used, and analyzed using methods known in the art. (See, e.g.,

Brennan, T.M. et al. (1995) U.S. Patent No. 5,474,796; Schena, M. et al. (1996) Proc. Natl. Acad. Sci. USA 93:10614-10619; Baldeschweiler et al. (1995) PCT application WO95/251116; Shalon, D. et al. (1995) PCT application WO95/35505; Heller, R.A. et al. (1997) Proc. Natl. Acad. Sci. USA 94:2150-2155; and Heller, M.J. et al. (1997) U.S. Patent No. 5,605,662.) Various types of microarrays are well known and thoroughly described in DNA Microarrays: A Practical Approach, M. Schena, ed. (1999) Oxford University Press, London, hereby expressly incorporated by reference.

In another embodiment of the invention, nucleic acid sequences encoding CDIFF may be used to generate hybridization probes useful in mapping the naturally occurring genomic sequence. Either coding or noncoding sequences may be used, and in some instances, noncoding sequences may be preferable over coding sequences. For example, conservation of a coding sequence among members of a multi-gene family may potentially cause undesired cross hybridization during chromosomal mapping. The sequences may be mapped to a particular chromosome, to a specific region of a chromosome, or to artificial chromosome constructions, e.g., human artificial chromosomes (HACs), yeast artificial chromosomes (YACs), bacterial artificial chromosomes (BACs), bacterial P1 constructions, or single chromosome cDNA libraries. (See, e.g., Harrington, J.J. et al. (1997) Nat. Genet. 15:345-355; Price, C.M. (1993) Blood Rev. 7:127-134; and Trask, B.J. (1991) Trends Genet. 7:149-154.) Once mapped, the nucleic acid sequences of the invention may be used to develop genetic linkage maps, for example, which correlate the inheritance of a disease state with the inheritance of a particular chromosome region or restriction fragment length polymorphism (RFLP). (See, e.g., Lander, E.S. and D. Botstein (1986) Proc. Natl. Acad. Sci. USA 83:7353-7357.)

Fluorescent in situ hybridization (FISH) may be correlated with other physical and genetic map data. (See, e.g., Heinz-Ulrich, et al. (1995) in Meyers, supra, pp. 965-968.) Examples of genetic map data can be found in various scientific journals or at the Online Mendelian Inheritance in Man (OMIM) World Wide Web site. Correlation between the location of the gene encoding CDIFF on a physical map and a specific disorder, or a predisposition to a specific disorder, may help define the region of DNA associated with that disorder and thus may further positional cloning efforts.

In situ hybridization of chromosomal preparations and physical mapping techniques, such as linkage analysis using established chromosomal markers, may be used for extending genetic maps. Often the placement of a gene on the chromosome of another mammalian species, such as mouse, may reveal associated markers even if the exact chromosomal locus is not known. This information is valuable to investigators searching for disease genes using positional cloning or other gene discovery techniques. Once the gene or genes responsible for a disease or syndrome have been crudely localized by genetic linkage to a particular genomic region, e.g., ataxia-telangiectasia to 11q22-23, any sequences mapping to that area may represent associated or regulatory genes for further investigation. (See, e.g., Gatti, R.A. et al. (1988) Nature 336:577-580.) The nucleotide sequence of

the instant invention may also be used to detect differences in the chromosomal location due to translocation, inversion, etc., among normal, carrier, or affected individuals.

In another embodiment of the invention, CDIFF, its catalytic or immunogenic fragments, or oligopeptides thereof can be used for screening libraries of compounds in any of a variety of drug screening techniques. The fragment employed in such screening may be free in solution, affixed to a solid support, borne on a cell surface, or located intracellularly. The formation of binding complexes between CDIFF and the agent being tested may be measured.

Another technique for drug screening provides for high throughput screening of compounds having suitable binding affinity to the protein of interest. (See, e.g., Geysen, et al. (1984) PCT application WO84/03564.) In this method, large numbers of different small test compounds are synthesized on a solid substrate. The test compounds are reacted with CDIFF, or fragments thereof, and washed. Bound CDIFF is then detected by methods well known in the art. Purified CDIFF can also be coated directly onto plates for use in the aforementioned drug screening techniques. Alternatively, non-neutralizing antibodies can be used to capture the peptide and immobilize it on a solid support.

In another embodiment, one may use competitive drug screening assays in which neutralizing antibodies capable of binding CDIFF specifically compete with a test compound for binding CDIFF. In this manner, antibodies can be used to detect the presence of any peptide which shares one or more antigenic determinants with CDIFF.

In additional embodiments, the nucleotide sequences which encode CDIFF may be used in any molecular biology techniques that have yet to be developed, provided the new techniques rely on properties of nucleotide sequences that are currently known, including, but not limited to, such properties as the triplet genetic code and specific base pair interactions.

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The following preferred specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever.

The disclosures of all patents, applications and publications, mentioned above and below, are hereby expressly incorporated by reference.

EXAMPLES

I. Construction of cDNA Libraries

RNA was purchased from Clontech or isolated from tissues described in Table 4. Some tissues were homogenized and lysed in guanidinium isothiocyanate, while others were homogenized and lysed in phenol or in a suitable mixture of denaturants, such as TRIZOL (Life Technologies), a

monophasic solution of phenol and guanidine isothiocyanate. The resulting lysates were centrifuged over CsCl cushions or extracted with chloroform. RNA was precipitated from the lysates with either isopropanol or sodium acetate and ethanol, or by other routine methods.

Phenol extraction and precipitation of RNA were repeated as necessary to increase RNA purity. In some cases, RNA was treated with DNase. For most libraries, poly(A+) RNA was isolated using oligo d(T)-coupled paramagnetic particles (Promega), OLIGOTEX latex particles (QIAGEN, Chatsworth CA), or an OLIGOTEX mRNA purification kit (QIAGEN). Alternatively, RNA was isolated directly from tissue lysates using other RNA isolation kits, e.g., the POLY(A)PURE mRNA purification kit (Ambion, Austin TX).

In some cases, Stratagene was provided with RNA and constructed the corresponding cDNA libraries. Otherwise, cDNA was synthesized and cDNA libraries were constructed with the UNIZAP vector system (Stratagene) or SUPERSCRIPT plasmid system (Life Technologies), using the recommended procedures or similar methods known in the art. (See, e.g., Ausubel, 1997, <u>supra</u>, units 5.1-6.6.) Reverse transcription was initiated using oligo d(T) or random primers. Synthetic oligonucleotide adapters were ligated to double stranded cDNA, and the cDNA was digested with the appropriate restriction enzyme or enzymes. For most libraries, the cDNA was size-selected (300-1000 bp) using SEPHACRYL S1000, SEPHAROSE CL2B, or SEPHAROSE CL4B column chromatography (Amersham Pharmacia Biotech) or preparative agarose gel electrophoresis. cDNAs were ligated into compatible restriction enzyme sites of the polylinker of a suitable plasmid, e.g., PBLUESCRIPT plasmid (Stratagene), PSPORT1 plasmid (Life Technologies), pcDNA2.1 plasmid (Invitrogen, Carlsbad CA), or pINCY plasmid (Incyte Genomics, Palo Alto CA). Recombinant plasmids were transformed into competent <u>E. coli</u> cells including XL1-Blue, XL1-BlueMRF, or SOLR from Stratagene or DH5α, DH10B, or ElectroMAX DH10B from Life Technologies.

II. Isolation of cDNA Clones

Plasmids obtained as described in Example I were recovered from host cells by <u>in vivo</u> excision using the UNIZAP vector system (Stratagene) or by cell lysis. Plasmids were purified using at least one of the following: a Magic or WIZARD Minipreps DNA purification system (Promega); an AGTC Miniprep purification kit (Edge Biosystems, Gaithersburg MD); and QIAWELL 8 Plasmid, QIAWELL 8 Plus Plasmid, QIAWELL 8 Ultra Plasmid purification systems or the R.E.A.L. PREP 96 plasmid purification kit from QIAGEN. Following precipitation, plasmids were resuspended in 0.1 ml of distilled water and stored, with or without lyophilization, at 4°C.

Alternatively, plasmid DNA was amplified from host cell lysates using direct link PCR in a high-throughput format (Rao, V.B. (1994) Anal. Biochem. 216:1-14). Host cell lysis and thermal cycling steps were carried out in a single reaction mixture. Samples were processed and stored in 384-well plates, and the concentration of amplified plasmid DNA was quantified fluorometrically

using PICOGREEN dye (Molecular Probes, Eugene OR) and a FLUOROSKAN II fluorescence scanner (Labsystems Oy, Helsinki, Finland).

III. Sequencing and Analysis

Incyte cDNA recovered in plasmids as described in Example II were sequenced as follows. Sequencing reactions were processed using standard methods or high-throughput instrumentation such as the ABI CATALYST 800 (PE Biosystems) thermal cycler or the PTC-200 thermal cycler (MJ Research) in conjunction with the HYDRA microdispenser (Robbins Scientific) or the MICROLAB 2200 (Hamilton) liquid transfer system. cDNA sequencing reactions were prepared using reagents provided by Amersham Pharmacia Biotech or supplied in ABI sequencing kits such as the ABI PRISM BIGDYE Terminator cycle sequencing ready reaction kit (PE Biosystems). Electrophoretic separation of cDNA sequencing reactions and detection of labeled polynucleotides were carried out using the MEGABACE 1000 DNA sequencing system (Molecular Dynamics); the ABI PRISM 373 or 377 sequencing system (PE Biosystems) in conjunction with standard ABI protocols and base calling software; or other sequence analysis systems known in the art. Reading frames within the cDNA sequences were identified using standard methods (reviewed in Ausubel, 1997, supra, unit 7.7). Some of the cDNA sequences were selected for extension using the techniques disclosed in Example VI.

The polynucleotide sequences derived from cDNA sequencing were assembled and analyzed using a combination of software programs which utilize algorithms well known to those skilled in the art. Table 5 summarizes the tools, programs, and algorithms used and provides applicable descriptions, references, and threshold parameters. The first column of Table 5 shows the tools, programs, and algorithms used, the second column provides brief descriptions thereof, the third column presents appropriate references, all of which are incorporated by reference herein in their entirety, and the fourth column presents, where applicable, the scores, probability values, and other parameters used to evaluate the strength of a match between two sequences (the higher the score, the greater the homology between two sequences). Sequences were analyzed using MACDNASIS PRO software (Hitachi Software Engineering, South San Francisco CA) and LASERGENE software (DNASTAR). Polynucleotide and polypeptide sequence alignments were generated using the default parameters specified by the clustal algorithm as incorporated into the MEGALIGN multisequence alignment program (DNASTAR), which also calculates the percent identity between aligned sequences.

The polynucleotide sequences were validated by removing vector, linker, and polyA sequences and by masking ambiguous bases, using algorithms and programs based on BLAST, dynamic programing, and dinucleotide nearest neighbor analysis. The sequences were then queried against a selection of public databases such as the GenBank primate, rodent, mammalian, vertebrate,

and eukaryote databases, and BLOCKS, PRINTS, DOMO, PRODOM, and PFAM to acquire annotation using programs based on BLAST, FASTA, and BLIMPS. The sequences were assembled into full length polynucleotide sequences using programs based on Phred, Phrap, and Consed, and were screened for open reading frames using programs based on GeneMark, BLAST, and FASTA. The full length polynucleotide sequences were translated to derive the corresponding full length amino acid sequences, and these full length sequences were subsequently analyzed by querying against databases such as the GenBank databases (described above), SwissProt, BLOCKS, PRINTS, DOMO, PRODOM, Prosite, and Hidden Markov Model (HMM)-based protein family databases such as PFAM. HMM is a probabilistic approach which analyzes consensus primary structures of gene families. (See, e.g., Eddy, S.R. (1996) Curr. Opin. Struct. Biol. 6:361-365.)

The programs described above for the assembly and analysis of full length polynucleotide and amino acid sequences were also used to identify polynucleotide sequence fragments from SEQ ID NO:29-56. Fragments from about 20 to about 4000 nucleotides which are useful in hybridization and amplification technologies were described in The Invention section above.

IV. Analysis of Polynucleotide Expression

Northern analysis is a laboratory technique used to detect the presence of a transcript of a gene and involves the hybridization of a labeled nucleotide sequence to a membrane on which RNAs from a particular cell type or tissue have been bound. (See, e.g., Sambrook, supra, ch. 7; Ausubel, 1995, supra, ch. 4 and 16.)

Analogous computer techniques applying BLAST were used to search for identical or related molecules in cDNA databases such as GenBank or LIFESEQ (Incyte Genomics). This analysis is much faster than multiple membrane-based hybridizations. In addition, the sensitivity of the computer search can be modified to determine whether any particular match is categorized as exact or similar. The basis of the search is the product score, which is defined as:

$$\frac{\text{BLAST Score x Percent Identity}}{\text{5 x minimum \{length(Seq. 1), length(Seq. 2)\}}}$$

The product score takes into account both the degree of similarity between two sequences and the length of the sequence match. The product score is a normalized value between 0 and 100, and is calculated as follows: the BLAST score is multiplied by the percent nucleotide identity and the product is divided by (5 times the length of the shorter of the two sequences). The BLAST score is calculated by assigning a score of +5 for every base that matches in a high-scoring segment pair (HSP), and -4 for every mismatch. Two sequences may share more than one HSP (separated by gaps). If there is more than one HSP, then the pair with the highest BLAST score is used to calculate the product score. The product score represents a balance between fractional overlap and quality in a

BLAST alignment. For example, a product score of 100 is produced only for 100% identity over the entire length of the shorter of the two sequences being compared. A product score of 70 is produced either by 100% identity and 70% overlap at one end, or by 88% identity and 100% overlap at the other. A product score of 50 is produced either by 100% identity and 50% overlap at one end, or 79% identity and 100% overlap.

The results of northern analyses are reported as a percentage distribution of libraries in which the transcript encoding CDIFF occurred. Analysis involved the categorization of cDNA libraries by organ/tissue and disease. The organ/tissue categories included cardiovascular, dermatologic, developmental, endocrine, gastrointestinal, hematopoietic/immune, musculoskeletal, nervous, reproductive, and urologic. The disease/condition categories included cancer, inflammation, trauma, cell proliferation, neurological, and pooled. For each category, the number of libraries expressing the sequence of interest was counted and divided by the total number of libraries across all categories. Percentage values of tissue-specific and disease- or condition-specific expression are reported in Table 3.

V. Chromosomal Mapping of CDIFF Encoding Polynucleotides

The sequences which were used to assemble SEQ ID NO:29-56 were compared with sequences from the Incyte LIFESEQ database and public domain databases using BLAST and other implementations of the Smith-Waterman algorithm. Sequences from these databases that matched SEQ ID NO:29-56 were assembled into clusters of contiguous and overlapping sequences using assembly algorithms such as Phrap (Table 5). Radiation hybrid and genetic mapping data available from public resources such as the Stanford Human Genome Center (SHGC), Whitehead Institute for Genome Research (WIGR), and Généthon were used to determine if any of the clustered sequences had been previously mapped. Inclusion of a mapped sequence in a cluster resulted in the assignment of all sequences of that cluster, including its particular SEQ ID NO:, to that map location.

Map locations are represented by ranges, or intervals, or human chromosomes. The map position of an interval, in centiMorgans, is measured relative to the terminus of the chromosome's p-arm. (The centiMorgan (cM) is a unit of measurement based on recombination frequencies between chromosomal markers. On average, 1 cM is roughly equivalent to 1 megabase (Mb) of DNA in humans, although this can vary widely due to hot and cold spots of recombination.) The cM distances are based on genetic markers mapped by Généthon which provide boundaries for radiation hybrid markers whose sequences were included in each of the clusters. Human genome maps and other resources available to the public, such as the NCBI "GeneMap'99" World Wide Web site (http://www.ncbi.nlm.nih.gov/genemap/), can be employed to determine if previously identified disease genes map within or in proximity to the intervals indicated above.

In this manner, SEQ ID NO:32 maps to chromosome 1 within the interval from 152.2 to

157.4 centiMorgans, to chromosome 3 within the interval from 157.4 to 158.0 centiMorgans, and to the X chromosome within the interval from 104.9 to 150.3 centiMorgans. The interval on chromosome 1 from 152.2 to 157.4 centiMorgans also contains genes associated with leukemia, hypothyroidism, and adrenal hyperplasia. The interval on the X chromosome from 104.9 to 150.3 centiMorgans also contains genes associated with X-linked lissencephaly, leiomyomatosis with Alport syndrome, lymphoproliferative syndrome, Bruton agammaglobulinemia, and diffuse angiokeratoma. SEQ ID NO:37 maps to chromosome 11 within the interval from 19.6 to 23.2 centiMorgans. SEQ ID NO:39 maps to chromosome 16 within the interval from 109.1 to 130.8 centiMorgans, and to chromosome 22 within the interval from 45.5 to 58.1 centiMorgans. The interval on chromosome 16 from 109.1 to 130.8 centiMorgans also contains a gene associated with gastric cancer susceptibility. SEQ ID NO:45 maps to chromosome 7 within the interval from 105.2 to 109.0 centiMorgans, to chromosome 17 within the interval from 65.0 to 90.2 centiMorgans, and to chromosome 20 within the interval from 50.2 to 54.9 centiMorgans. The interval on chromosome 7 from 105.2 to 109.0 centiMorgans also contains a gene associated with osteogenesis imperfecta. The interval on chromosome 17 from 65.0 to 90.2 centiMorgans also contains genes associated with breast cancer, hepatic leukemia, myeloperoxidase deficiency, muscular dystrophy, periodic paralysis, and placental growth. SEQ ID NO:54 maps to chromosome 12 within the interval from 21.3 to 36.1 centiMorgans. SEQ ID NO:55 maps to chromosome 1 within the interval from 22.9 to 39.9 centiMorgans and to chromosome 3 within the interval from 30.9 to 43.0 centiMorgans.

More than one map location is reported for SEQ ID NO:32, SEQ ID NO:39, SEQ ID NO:45, and SEQ ID NO:55, indicating that sequences having different map locations were assembled into a single cluster. This situation occurs, for example, when sequences having strong similarity, but not complete identity, are assembled into a single cluster.

VI. Extension of CDIFF Encoding Polynucleotides

The full length nucleic acid sequences of SEQ ID NO:29-56 were produced by extension of an appropriate fragment of the full length molecule using oligonucleotide primers designed from this fragment. One primer was synthesized to initiate 5' extension of the known fragment, and the other primer, to initiate 3' extension of the known fragment. The initial primers were designed using OLIGO 4.06 software (National Biosciences), or another appropriate program, to be about 22 to 30 nucleotides in length, to have a GC content of about 50% or more, and to anneal to the target sequence at temperatures of about 68°C to about 72°C. Any stretch of nucleotides which would result in hairpin structures and primer-primer dimerizations was avoided.

Selected human cDNA libraries were used to extend the sequence. If more than one extension was necessary or desired, additional or nested sets of primers were designed.

High fidelity amplification was obtained by PCR using methods well known in the art. PCR

was performed in 96-well plates using the PTC-200 thermal cycler (MJ Research, Inc.). The reaction mix contained DNA template, 200 nmol of each primer, reaction buffer containing Mg^{2+}, $(NH_4)_2SO_4$, and β-mercaptoethanol, Taq DNA polymerase (Amersham Pharmacia Biotech), ELONGASE enzyme (Life Technologies), and Pfu DNA polymerase (Stratagene), with the following parameters for primer pair PCI A and PCI B: Step 1: 94°C, 3 min; Step 2: 94°C, 15 sec; Step 3: 60°C, 1 min; Step 4: 68°C, 2 min; Step 5: Steps 2, 3, and 4 repeated 20 times; Step 6: 68°C, 5 min; Step 7: storage at 4°C. In the alternative, the parameters for primer pair T7 and SK+ were as follows: Step 1: 94°C, 3 min; Step 2: 94°C, 15 sec; Step 3: 57°C, 1 min; Step 4: 68°C, 2 min; Step 5: Steps 2, 3, and 4 repeated 20 times; Step 6: 68°C, 5 min; Step 7: storage at 4°C.

The concentration of DNA in each well was determined by dispensing 100 μl PICOGREEN quantitation reagent (0.25% (v/v) PICOGREEN; Molecular Probes, Eugene OR) dissolved in 1X TE and 0.5 μl of undiluted PCR product into each well of an opaque fluorimeter plate (Corning Costar, Acton MA), allowing the DNA to bind to the reagent. The plate was scanned in a Fluoroskan II (Labsystems Oy, Helsinki, Finland) to measure the fluorescence of the sample and to quantify the concentration of DNA. A 5 μl to 10 μl aliquot of the reaction mixture was analyzed by electrophoresis on a 1 % agarose mini-gel to determine which reactions were successful in extending the sequence.

The extended nucleotides were desalted and concentrated, transferred to 384-well plates, digested with CviJI cholera virus endonuclease (Molecular Biology Research, Madison WI), and sonicated or sheared prior to religation into pUC 18 vector (Amersham Pharmacia Biotech). For shotgun sequencing, the digested nucleotides were separated on low concentration (0.6 to 0.8%) agarose gels, fragments were excised, and agar digested with Agar ACE (Promega). Extended clones were religated using T4 ligase (New England Biolabs, Beverly MA) into pUC 18 vector (Amersham Pharmacia Biotech), treated with Pfu DNA polymerase (Stratagene) to fill-in restriction site overhangs, and transfected into competent E. coli cells. Transformed cells were selected on antibiotic-containing media, and individual colonies were picked and cultured overnight at 37°C in 384-well plates in LB/2x carb liquid media.

The cells were lysed, and DNA was amplified by PCR using Taq DNA polymerase (Amersham Pharmacia Biotech) and Pfu DNA polymerase (Stratagene) with the following parameters: Step 1: 94°C, 3 min; Step 2: 94°C, 15 sec; Step 3: 60°C, 1 min; Step 4: 72°C, 2 min; Step 5: steps 2, 3, and 4 repeated 29 times; Step 6: 72°C, 5 min; Step 7: storage at 4°C. DNA was quantified by PICOGREEN reagent (Molecular Probes) as described above. Samples with low DNA recoveries were reamplified using the same conditions as described above. Samples were diluted with 20% dimethysulfoxide (1:2, v/v), and sequenced using DYENAMIC energy transfer sequencing primers and the DYENAMIC DIRECT kit (Amersham Pharmacia Biotech) or the ABI PRISM

BIGDYE Terminator cycle sequencing ready reaction kit (PE Biosystems).

In like manner, the polynucleotide sequences of SEQ ID NO:29-56 are used to obtain 5' regulatory sequences using the procedure above, along with oligonucleotides designed for such extension, and an appropriate genomic library.

VII. Labeling and Use of Individual Hybridization Probes

Hybridization probes derived from SEQ ID NO:29-56 are employed to screen cDNAs, genomic DNAs, or mRNAs. Although the labeling of oligonucleotides, consisting of about 20 base pairs, is specifically described, essentially the same procedure is used with larger nucleotide fragments. Oligonucleotides are designed using state-of-the-art software such as OLIGO 4.06 software (National Biosciences) and labeled by combining 50 pmol of each oligomer, 250 μCi of [γ-^{32}P] adenosine triphosphate (Amersham Pharmacia Biotech), and T4 polynucleotide kinase (DuPont NEN, Boston MA). The labeled oligonucleotides are substantially purified using a SEPHADEX G-25 superfine size exclusion dextran bead column (Amersham Pharmacia Biotech). An aliquot containing 10^7 counts per minute of the labeled probe is used in a typical membrane-based hybridization analysis of human genomic DNA digested with one of the following endonucleases: Ase I, Bgl II, Eco RI, Pst I, Xba I, or Pvu II (DuPont NEN).

The DNA from each digest is fractionated on a 0.7% agarose gel and transferred to nylon membranes (Nytran Plus, Schleicher & Schuell, Durham NH). Hybridization is carried out for 16 hours at 40°C. To remove nonspecific signals, blots are sequentially washed at room temperature under conditions of up to, for example, 0.1 x saline sodium citrate and 0.5% sodium dodecyl sulfate. Hybridization patterns are visualized using autoradiography or an alternative imaging means and compared.

VIII. Microarrays

The linkage or synthesis of array elements upon a microarray can be achieved utilizing photolithography, piezoelectric printing (ink-jet printing, See, e.g., Baldeschweiler, supra), mechanical microspotting technologies, and derivatives thereof. The substrate in each of the aforementioned technologies should be uniform and solid with a non-porous surface (Schena (1999), supra). Suggested substrates include silicon, silica, glass slides, glass chips, and silicon wafers. Alternatively, a procedure analogous to a dot or slot blot may also be used to arrange and link elements to the surface of a substrate using thermal, UV, chemical, or mechanical bonding procedures. A typical array may be produced using available methods and machines well known to those of ordinary skill in the art and may contain any appropriate number of elements. (See, e.g., Schena, M. et al. (1995) Science 270:467-470; Shalon, D. et al. (1996) Genome Res. 6:639-645; Marshall, A. and J. Hodgson (1998) Nat. Biotechnol. 16:27-31.)

Full length cDNAs, Expressed Sequence Tags (ESTs), or fragments or oligomers thereof may

comprise the elements of the microarray. Fragments or oligomers suitable for hybridization can be selected using software well known in the art such as LASERGENE software (DNASTAR). The array elements are hybridized with polynucleotides in a biological sample. The polynucleotides in the biological sample are conjugated to a fluorescent label or other molecular tag for ease of detection. After hybridization, nonhybridized nucleotides from the biological sample are removed, and a fluorescence scanner is used to detect hybridization at each array element. Alternatively, laser desorbtion and mass spectrometry may be used for detection of hybridization. The degree of complementarity and the relative abundance of each polynucleotide which hybridizes to an element on the microarray may be assessed. In one embodiment, microarray preparation and usage is described in detail below.

Tissue or Cell Sample Preparation

Total RNA is isolated from tissue samples using the guanidinium thiocyanate method and poly(A)$^+$ RNA is purified using the oligo-(dT) cellulose method. Each poly(A)$^+$ RNA sample is reverse transcribed using MMLV reverse-transcriptase, 0.05 pg/µl oligo-(dT) primer (21mer), 1X first strand buffer, 0.03 units/µl RNase inhibitor, 500 µM dATP, 500 µM dGTP, 500 µM dTTP, 40 µM dCTP, 40 µM dCTP-Cy3 (BDS) or dCTP-Cy5 (Amersham Pharmacia Biotech). The reverse transcription reaction is performed in a 25 ml volume containing 200 ng poly(A)$^+$ RNA with GEMBRIGHT kits (Incyte). Specific control poly(A)$^+$ RNAs are synthesized by in vitro transcription from non-coding yeast genomic DNA. After incubation at 37 °C for 2 hr, each reaction sample (one with Cy3 and another with Cy5 labeling) is treated with 2.5 ml of 0.5M sodium hydroxide and incubated for 20 minutes at 85 °C to the stop the reaction and degrade the RNA. Samples are purified using two successive CHROMA SPIN 30 gel filtration spin columns (CLONTECH Laboratories, Inc. (CLONTECH), Palo Alto CA) and after combining, both reaction samples are ethanol precipitated using 1 ml of glycogen (1 mg/ml), 60 ml sodium acetate, and 300 ml of 100% ethanol. The sample is then dried to completion using a SpeedVAC (Savant Instruments Inc., Holbrook NY) and resuspended in 14 µl 5X SSC/0.2% SDS.

Microarray Preparation

Sequences of the present invention are used to generate array elements. Each array element is amplified from bacterial cells containing vectors with cloned cDNA inserts. PCR amplification uses primers complementary to the vector sequences flanking the cDNA insert. Array elements are amplified in thirty cycles of PCR from an initial quantity of 1-2 ng to a final quantity greater than 5 µg. Amplified array elements are then purified using SEPHACRYL-400 (Amersham Pharmacia Biotech).

Purified array elements are immobilized on polymer-coated glass slides. Glass microscope slides (Corning) are cleaned by ultrasound in 0.1% SDS and acetone, with extensive distilled water

washes between and after treatments. Glass slides are etched in 4% hydrofluoric acid (VWR Scientific Products Corporation (VWR), West Chester PA), washed extensively in distilled water, and coated with 0.05% aminopropyl silane (Sigma) in 95% ethanol. Coated slides are cured in a 110°C oven.

Array elements are applied to the coated glass substrate using a procedure described in US Patent No. 5,807,522, incorporated herein by reference. 1 µl of the array element DNA, at an average concentration of 100 ng/µl, is loaded into the open capillary printing element by a high-speed robotic apparatus. The apparatus then deposits about 5 nl of array element sample per slide.

Microarrays are UV-crosslinked using a STRATALINKER UV-crosslinker (Stratagene). Microarrays are washed at room temperature once in 0.2% SDS and three times in distilled water. Non-specific binding sites are blocked by incubation of microarrays in 0.2% casein in phosphate buffered saline (PBS) (Tropix, Inc., Bedford MA) for 30 minutes at 60 °C followed by washes in 0.2% SDS and distilled water as before.

Hybridization

Hybridization reactions contain 9 µl of sample mixture consisting of 0.2 µg each of Cy3 and Cy5 labeled cDNA synthesis products in 5X SSC, 0.2% SDS hybridization buffer. The sample mixture is heated to 65 °C for 5 minutes and is aliquoted onto the microarray surface and covered with an 1.8 cm^2 coverslip. The arrays are transferred to a waterproof chamber having a cavity just slightly larger than a microscope slide. The chamber is kept at 100% humidity internally by the addition of 140 µl of 5X SSC in a corner of the chamber. The chamber containing the arrays is incubated for about 6.5 hours at 60 °C. The arrays are washed for 10 min at 45 °C in a first wash buffer (1X SSC, 0.1% SDS), three times for 10 minutes each at 45 °C in a second wash buffer (0.1X SSC), and dried.

Detection

Reporter-labeled hybridization complexes are detected with a microscope equipped with an Innova 70 mixed gas 10 W laser (Coherent, Inc., Santa Clara CA) capable of generating spectral lines at 488 nm for excitation of Cy3 and at 632 nm for excitation of Cy5. The excitation laser light is focused on the array using a 20X microscope objective (Nikon, Inc., Melville NY). The slide containing the array is placed on a computer-controlled X-Y stage on the microscope and raster-scanned past the objective. The 1.8 cm x 1.8 cm array used in the present example is scanned with a resolution of 20 micrometers.

In two separate scans, a mixed gas multiline laser excites the two fluorophores sequentially. Emitted light is split, based on wavelength, into two photomultiplier tube detectors (PMT R1477, Hamamatsu Photonics Systems, Bridgewater NJ) corresponding to the two fluorophores. Appropriate filters positioned between the array and the photomultiplier tubes are used to filter the signals. The

emission maxima of the fluorophores used are 565 nm for Cy3 and 650 nm for Cy5. Each array is typically scanned twice, one scan per fluorophore using the appropriate filters at the laser source, although the apparatus is capable of recording the spectra from both fluorophores simultaneously.

The sensitivity of the scans is typically calibrated using the signal intensity generated by a cDNA control species added to the sample mixture at a known concentration. A specific location on the array contains a complementary DNA sequence, allowing the intensity of the signal at that location to be correlated with a weight ratio of hybridizing species of 1:100,000. When two samples from different sources (e.g., representing test and control cells), each labeled with a different fluorophore, are hybridized to a single array for the purpose of identifying genes that are differentially expressed, the calibration is done by labeling samples of the calibrating cDNA with the two fluorophores and adding identical amounts of each to the hybridization mixture.

The output of the photomultiplier tube is digitized using a 12-bit RTI-835H analog-to-digital (A/D) conversion board (Analog Devices, Inc., Norwood MA) installed in an IBM-compatible PC computer. The digitized data are displayed as an image where the signal intensity is mapped using a linear 20-color transformation to a pseudocolor scale ranging from blue (low signal) to red (high signal). The data is also analyzed quantitatively. Where two different fluorophores are excited and measured simultaneously, the data are first corrected for optical crosstalk (due to overlapping emission spectra) between the fluorophores using each fluorophore's emission spectrum.

A grid is superimposed over the fluorescence signal image such that the signal from each spot is centered in each element of the grid. The fluorescence signal within each element is then integrated to obtain a numerical value corresponding to the average intensity of the signal. The software used for signal analysis is the GEMTOOLS gene expression analysis program (Incyte).

IX. Complementary Polynucleotides

Sequences complementary to the CDIFF-encoding sequences, or any parts thereof, are used to detect, decrease, or inhibit expression of naturally occurring CDIFF. Although use of oligonucleotides comprising from about 15 to 30 base pairs is described, essentially the same procedure is used with smaller or with larger sequence fragments. Appropriate oligonucleotides are designed using OLIGO 4.06 software (National Biosciences) and the coding sequence of CDIFF. To inhibit transcription, a complementary oligonucleotide is designed from the most unique 5' sequence and used to prevent promoter binding to the coding sequence. To inhibit translation, a complementary oligonucleotide is designed to prevent ribosomal binding to the CDIFF-encoding transcript.

X. Expression of CDIFF

Expression and purification of CDIFF is achieved using bacterial or virus-based expression systems. For expression of CDIFF in bacteria, cDNA is subcloned into an appropriate vector

containing an antibiotic resistance gene and an inducible promoter that directs high levels of cDNA transcription. Examples of such promoters include, but are not limited to, the *trp-lac* (*tac*) hybrid promoter and the T5 or T7 bacteriophage promoter in conjunction with the *lac* operator regulatory element. Recombinant vectors are transformed into suitable bacterial hosts, e.g., BL21(DE3). Antibiotic resistant bacteria express CDIFF upon induction with isopropyl beta-D-thiogalactopyranoside (IPTG). Expression of CDIFF in eukaryotic cells is achieved by infecting insect or mammalian cell lines with recombinant Autographica californica nuclear polyhedrosis virus (AcMNPV), commonly known as baculovirus. The nonessential polyhedrin gene of baculovirus is replaced with cDNA encoding CDIFF by either homologous recombination or bacterial-mediated transposition involving transfer plasmid intermediates. Viral infectivity is maintained and the strong polyhedrin promoter drives high levels of cDNA transcription. Recombinant baculovirus is used to infect Spodoptera frugiperda (Sf9) insect cells in most cases, or human hepatocytes, in some cases. Infection of the latter requires additional genetic modifications to baculovirus. (See Engelhard, E.K. et al. (1994) Proc. Natl. Acad. Sci. USA 91:3224-3227; Sandig, V. et al. (1996) Hum. Gene Ther. 7:1937-1945.)

In most expression systems, CDIFF is synthesized as a fusion protein with, e.g., glutathione S-transferase (GST) or a peptide epitope tag, such as FLAG or 6-His, permitting rapid, single-step, affinity-based purification of recombinant fusion protein from crude cell lysates. GST, a 26-kilodalton enzyme from Schistosoma japonicum, enables the purification of fusion proteins on immobilized glutathione under conditions that maintain protein activity and antigenicity (Amersham Pharmacia Biotech). Following purification, the GST moiety can be proteolytically cleaved from CDIFF at specifically engineered sites. FLAG, an 8-amino acid peptide, enables immunoaffinity purification using commercially available monoclonal and polyclonal anti-FLAG antibodies (Eastman Kodak). 6-His, a stretch of six consecutive histidine residues, enables purification on metal-chelate resins (QIAGEN). Methods for protein expression and purification are discussed in Ausubel (1995, supra, ch. 10 and 16). Purified CDIFF obtained by these methods can be used directly in the assays shown in Examples XI and XV.

XI. Demonstration of CDIFF Activity

CDIFF activity is demonstrated by measuring the induction of terminal differentiation or cell cycle progression when CDIFF is expressed at physiologically elevated levels in mammalian cell culture systems. cDNA is subcloned into a mammalian expression vector containing a strong promoter that drives high levels of cDNA expression. Vectors of choice include pCMV SPORT™ (Life Technologies, Gaithersburg, MD) and pCR™ 3.1 (Invitrogen, Carlsbad, CA), both of which contain the cytomegalovirus promoter. 5-10 μg of recombinant vector are transiently transfected into a human cell line, preferably of endothelial or hematopoietic origin, using either liposome

formulations or electroporation. 1-2 μg of an additional plasmid containing sequences encoding a marker protein are co-transfected. Expression of a marker protein provides a means to distinguish transfected cells from nontransfected cells and is a reliable predictor of cDNA expression from the recombinant vector. Marker proteins of choice include, e.g., Green Fluorescent Protein (GFP) (Clontech, Palo Alto, CA), CD64, or a CD64-GFP fusion protein. Flow cytometry detects and quantifies the uptake of fluorescent molecules that diagnose events preceding or coincident with cell cycle progression or terminal differentiation. These events include changes in nuclear DNA content as measured by staining of DNA with propidium iodide; changes in cell size and granularity as measured by forward light scatter and 90 degree side light scatter; up or down-regulation of DNA synthesis as measured by decrease in bromodeoxyuridine uptake; alterations in expression of cell surface and intracellular proteins as measured by reactivity with specific antibodies; and alterations in plasma membrane composition as measured by the binding of fluorescein-conjugated Annexin V protein to the cell surface. Methods in flow cytometry are discussed in Ormerod, M. G. (1994) Flow Cytometry, Oxford, New York, NY.

XII. Functional Assays

CDIFF function is assessed by expressing the sequences encoding CDIFF at physiologically elevated levels in mammalian cell culture systems. cDNA is subcloned into a mammalian expression vector containing a strong promoter that drives high levels of cDNA expression. Vectors of choice include pCMV SPORT plasmid (Life Technologies) and pCR3.1 plasmid (Invitrogen), both of which contain the cytomegalovirus promoter. 5-10 μg of recombinant vector are transiently transfected into a human cell line, for example, an endothelial or hematopoietic cell line, using either liposome formulations or electroporation. 1-2 μg of an additional plasmid containing sequences encoding a marker protein are co-transfected. Expression of a marker protein provides a means to distinguish transfected cells from nontransfected cells and is a reliable predictor of cDNA expression from the recombinant vector. Marker proteins of choice include, e.g., Green Fluorescent Protein (GFP; Clontech), CD64, or a CD64-GFP fusion protein. Flow cytometry (FCM), an automated, laser optics-based technique, is used to identify transfected cells expressing GFP or CD64-GFP and to evaluate the apoptotic state of the cells and other cellular properties. FCM detects and quantifies the uptake of fluorescent molecules that diagnose events preceding or coincident with cell death. These events include changes in nuclear DNA content as measured by staining of DNA with propidium iodide; changes in cell size and granularity as measured by forward light scatter and 90 degree side light scatter; down-regulation of DNA synthesis as measured by decrease in bromodeoxyuridine uptake; alterations in expression of cell surface and intracellular proteins as measured by reactivity with specific antibodies; and alterations in plasma membrane composition as measured by the binding of fluorescein-conjugated Annexin V protein to the cell surface. Methods in flow cytometry are

discussed in Ormerod, M.G. (1994) Flow Cytometry, Oxford, New York NY.

The influence of CDIFF on gene expression can be assessed using highly purified populations of cells transfected with sequences encoding CDIFF and either CD64 or CD64-GFP. CD64 and CD64-GFP are expressed on the surface of transfected cells and bind to conserved regions of human immunoglobulin G (IgG). Transfected cells are efficiently separated from nontransfected cells using magnetic beads coated with either human IgG or antibody against CD64 (DYNAL, Lake Success NY). mRNA can be purified from the cells using methods well known by those of skill in the art. Expression of mRNA encoding CDIFF and other genes of interest can be analyzed by northern analysis or microarray techniques.

XIII. Production of CDIFF Specific Antibodies

CDIFF substantially purified using polyacrylamide gel electrophoresis (PAGE; see, e.g., Harrington, M.G. (1990) Methods Enzymol. 182:488-495), or other purification techniques, is used to immunize rabbits and to produce antibodies using standard protocols.

Alternatively, the CDIFF amino acid sequence is analyzed using LASERGENE software (DNASTAR) to determine regions of high immunogenicity, and a corresponding oligopeptide is synthesized and used to raise antibodies by means known to those of skill in the art. Methods for selection of appropriate epitopes, such as those near the C-terminus or in hydrophilic regions are well described in the art. (See, e.g., Ausubel, 1995, supra, ch. 11.)

Typically, oligopeptides of about 15 residues in length are synthesized using an ABI 431A peptide synthesizer (PE Biosystems) using FMOC chemistry and coupled to KLH (Sigma-Aldrich, St. Louis MO) by reaction with N-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) to increase immunogenicity. (See, e.g., Ausubel, 1995, supra.) Rabbits are immunized with the oligopeptide-KLH complex in complete Freund's adjuvant. Resulting antisera are tested for antipeptide and anti-CDIFF activity by, for example, binding the peptide or CDIFF to a substrate, blocking with 1% BSA, reacting with rabbit antisera, washing, and reacting with radio-iodinated goat anti-rabbit IgG.

XIV. Purification of Naturally Occurring CDIFF Using Specific Antibodies

Naturally occurring or recombinant CDIFF is substantially purified by immunoaffinity chromatography using antibodies specific for CDIFF. An immunoaffinity column is constructed by covalently coupling anti-CDIFF antibody to an activated chromatographic resin, such as CNBr-activated SEPHAROSE (Amersham Pharmacia Biotech). After the coupling, the resin is blocked and washed according to the manufacturer's instructions.

Media containing CDIFF are passed over the immunoaffinity column, and the column is washed under conditions that allow the preferential absorbance of CDIFF (e.g., high ionic strength buffers in the presence of detergent). The column is eluted under conditions that disrupt antibody/CDIFF binding (e.g., a buffer of pH 2 to pH 3, or a high concentration of a chaotrope, such

as urea or thiocyanate ion), and CDIFF is collected.

XV. Identification of Molecules Which Interact with CDIFF

CDIFF, or biologically active fragments thereof, are labeled with ^{125}I Bolton-Hunter reagent. (See, e.g., Bolton A.E. and W.M. Hunter (1973) Biochem. J. 133:529-539.) Candidate molecules previously arrayed in the wells of a multi-well plate are incubated with the labeled CDIFF, washed, and any wells with labeled CDIFF complex are assayed. Data obtained using different concentrations of CDIFF are used to calculate values for the number, affinity, and association of CDIFF with the candidate molecules.

CDIFF may also be used in the PATHCALLING process (CuraGen Corp., New Haven CT) which employs the yeast two-hybrid system in a high-throughput manner to determine all interactions between the proteins encoded by two large libraries of genes (Nandabalan, K. et al. (2000) U.S. Patent No. 6,057,101).

Various modifications and variations of the described methods and systems of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with certain embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

Table 1

Polypeptide SEQ ID NO:	Nucleotide SEQ ID NO:	Clone ID	Library	Fragments
1	29	1681724	STOMFET01	926756T1 (BRAINOT04), 1681724F7 (STOMFET01), 3335675F6 (BRAIFET01), 4182048H1 (BRAUNOT01)
2	30	1718047	UCMCNOT02	1714785H1 (UCMCNOT02), 1718047F6 (UCMCNOT02), 1718047H1 (UCMCNOT02)
3	31	1980323	LUNGTUT03	1321706H1 (BLADNOT04), 1361483F1 (LUNGNOT12), 1699071H1 (BLADTUT05), 1980323H1 (LUNGTUT03), 2015763H1 (ISLTNOT01)
4	32	1990956	CORPNOT02	640726F1 (BRSTNOT03), 1990956H1 (CORPNOT02), 2605626F6 (LUNGTUT07)
5	33	2009069	TESTNOT03	2009069H1 (TESTNOT03), 2009069R6 (TESTNOT03), g2077289
6	34	2009435	TESTNOT03	2009435H1 (TESTNOT03), SCWA00196V1, SXBC00564V1, SXBC00022V1, SXBC01668V1, SXBC01355V1, SCSA02588V1
7	35	2027937	KERANOT02	2027937H1 (KERANOT02), 2027937R6 (KERANOT02), 2027937T6 (KERANOT02), 3564727H1 (SKINNOT05)
8	36	2722347	LUNGTUT10	432586H1 (BRAVUNT02), 1415481H1 (BRAINOT12), 1435248X315V1 (PANCNOT08), 1749410F6 (STOMTUT02), 1853282F6 (LUNGFET03), 2722347H1 (LUNGTUT10), 2789091H1 (TLYMNOT03), 2832659H1 (TLYMNOT03), 2832889F6 (TLYMNOT03), 2832889T6 (TLYMNOT03), 2865507H1 (KIDNNOT20), 2963380H1 (SCORNOT04), 2999974H1 (TLYMNOT06), 3461418F6 (293TF2T01), 3496089H1 (ADRETUT07), 3584788H1 (293TF4T01), 4177153H1 (BRAINOT22), 4753938H1 (BRAHNOT01), 4755628H1 (BRAHNOT01), 5376660H1 (BRAXNOT01), 5401439H1 (BRAHNOT01), 5541291H1
9	37	2759876	THP1AZS08	1629112T6 (COLNPOT01), 2579860H2 (KIDNTUT13), 2759876H1 (THP1AZS08), 3222301H1 (COLNNON03)
10	38	2763735	BRSTNOT12	2505847X315F1 (CONUTUT01), 2505847X321F1 (CONUTUT01), 2763735H1 (BRSTNOT12), 2763735T6 (BRSTNOT12), SXRA00601V1, SXRA00968V1, SXRA00228V1
11	39	2848676	BRSTTUT13	734817R1 (TONSNOT01), 737069R6 (TONSNOT01), 1234242F1 (LUNGFET03), 1454710F1 (PENITUT01), 1720410T6 (BLADNOT06), 2635641F6 (BONTNOT01), 2674455H1 (KIDNNOT19), 2848676H1 (BRSTTUT13), 5279555H1 (MUSLNOT01), SAJA00783F1, SAJA01974F1
12	40	2956153	KIDNFET01	1413066F6 (BRAINOT12), 2290049R6 (BRAINON01), 2956153H1 (KIDNFET01), g802966
13	41	3333139	BRAIFET01	1758404R6 (PITUNOT03), 3030096F6 (HEARFET02), 3068943F6 (UTRSNOR01), 3333139H1 (BRAIFET01), SCBA00923V1
14	42	3432292	SKINNOT04	3432292F6 (SKINNOT04), 3432292H1 (SKINNOT04)

Table 1 (cont.)

Polypeptide SEQ ID NO:	Nucleotide SEQ ID NO:	Clone ID	Library	Fragments
15	43	3478571	OVARNOT11	1297225F6 (BRSTNOT07), 1297225X300D1 (BRSTNOT07), 2991617F6 (KIDNFET02), 3478571F6 (OVARNOT11), 3478571H1 (OVARNOT11), 3528528H1 (BLADNOT09), 4149136H1 (SINITUT04), 5272286T6 (OVARDIN02), SXZA00403V1
16	44	3495166	ADRETUT07	1648795H1 (PROSTUT09), 3495166H1 (ADRETUT07), 4530057H1 (LYMBTXT01), g3593882
17	45	3554748	SYNONOT01	1382675F1 (BRAITUT08), 2158036F6 (BRAINOT09), 3026430F6 (HEARFET02), 3026430T6 (HEARFET02), 3554748H1 (SYNONOT01), 3561906F6 (SKINNOT05), SBQA01579D1, SBQA03396D1, SBQA03390D1, SBQA04634D1, SBVA04060V1
18	46	3555629	LUNGNOT31	1665425F6 (BRSTNOT09), 1972328F6 (UCMCL5T01), 2415049F6 (HNT3AZT01), 2506456T6 (CONUTUT01), 3171395F6 (BRSTNOT18), 3251788H1 (SEMVNOT03), 3555629H1 (LUNGNOT31), 4764178H1 (PLACNOT05)
19	47	639636	BRSTNOT03	639636H1 (BRSTNOT03), 1301029T1 (BRSTNOT07), 3181614H1 (TLYJNOT01)
20	48	902218	BRSTTUT03	044115R6 (TBLYNOT01), 060192F1 (LUNGNOT01), 260135R1 (HNT2RAT01), 902218H1 (BRSTTUT03), 902218X312D1 (BRSTTUT03), 1223330R1 (COLNTUT02), 1282742F1 (COLNNOT16), 1867809F6 (SKINBIT01), 2630675F6 (COLNTUT15), 2725602F6 (OVARTUT05), 2822002F6 (ADRETUT06), g1444109
21	49	1360522	LUNGNOT12	1360389F6 (LUNGNOT12), 1360522H1 (LUNGNOT12), 1542022R1 (SINTTUT01), 2451247H1 (ENDANOT01)
22	50	1400678	BRAITUT08	1228811H1 (BRAITUT01), 1269918X305D1 (BRAINOT09), 1400678F6 (BRAITUT08), 1400678H1 (BRAITUT08), 1400678T6 (BRAITUT08), 1661858F6 (BRSTNOT09), 3119749H1 (LUNGTUT13), 3460603H1 (293TF1T01), 4751626F6 (BRAHNOT01), 5371724H1 (BRAINOT22), 5495045H1 (BRABDIR01)
23	51	1435556	PANCNOT08	1435556H1 (PANCNOT08), SCBA03895V1, SCBA01544V1, g1939223
24	52	1546633	PROSTUT04	1546633CT1 (PROSTUT04), 1546633H1 (PROSTUT04), 1834033R6 (BRAINON01), 2522443H1 (BRAITUT21)
25	53	1794031	PROSTUT05	1794031H1 (PROSTUT05), 2848189T6 (BRSTTUT13), 2950768F6 (KIDNFET01), 5261333F6 (CONDTUT01)
26	54	2060563	OVARNOT03	1209716R1 (BRSTNOT02), 1392224F1 (THYRNOT03), 2060563H1 (OVARNOT03), 2060563R6 (OVARNOT03), 3568425H1 (HEAPNOT01)

Table 1 (cont.)

Polypeptide SEQ ID NO:	Nucleotide SEQ ID NO:	Clone ID	Library	Fragments
27	55	2573955	HIPOAZT01	734802R1 (TONSNOT01), 1576203F6 (LNODNOT03), 1576203T6 (LNODNOT03), 1923163X305D2 (BRSTTUT01), 2125677X304D1 (BRSTNOT07), 2208587F6 (SINTFET03), 2573955H1 (HIPOAZT01), 3422039F6 (UCMCNOT04), 3422039T6 (UCMCNOT04), g1015103
28	56	3404792	ESOGNOT03	1267840H1 (BRAINOT09), 1599987F6 (BLADNOT03), 2814308F6 (OVARNOT10), 3404792H1 (ESOGNOT03)

Table 2

Polypeptide SEQ ID NO:	Amino Acid Residues	Potential Phosphorylation Sites	Potential Glycosylation Sites	Signature Sequence	Homologous Sequence	Analytical Methods and Databases
1	367	S18 S109 T267 S300 S58 S286	N11 N187	Signal peptide: M1-S58 Glutathione S-transferase domain: P43-F309	ganglioside-induced differentiation associated protein 1 [Mus musculus] g3378454	BLAST-GenBank MOTIFS SPSCAN HMMER-PFAM
2	102	S33 T39			erythroid differentiation-related factor [Mus musculus] g3003046	BLAST-GenBank MOTIFS
3	205	T51 S91 Y174			SOUL protein (eye and pineal gland sepcific gene product) [Mus musculus] g4886906	BLAST-GenBank MOTIFS
4	120	T94 S3 T29		HGR74/REX3 protein signature: N16-P120	REX-3 [Mus musculus] g3510643	BLAST-GenBank MOTIFS BLAST-PRODOM
5	108	S24 T33 T48 T49 S61		Spermatid DNA-binding protein domain: A2-E104	Spermatid nuclear transition protein [Sus scrofa] Q09821 (P-value=5.4x10-9)	BLAST-SwissProt MOTIFS BLAST-PRODOM
6	308	S72 T83 S100 T104 S137 T218 S241 T268 T42 S89 S92 S115 T165 T213 T228 S262	N70	Signal peptide: M1-S29	GSG1 (germ-cell specific gene) [Mus musculus] g4150939	BLAST-GenBank MOTIFS SPSCAN

Table 2 (Cont.)

Polypeptide SEQ ID NO:	Amino Acid Residues	Potential Phosphorylation Sites	Potential Glycosylation Sites	Signature Sequence	Homologous Sequence	Analytical Methods and Databases
7	116	S2 S60		Signal peptide: M1-C57 Small proline-rich proteins motif: S4-P16; Q80-C89	SPR2H protein (small proline-rich protein) [Mus musculus] g3093371	BLAST-GenBank MOTIFS SPSCAN BLIMPS-PRINTS
8	1253	T6 S12 T44 S68 S203 T256 S293 S367 S375 S382 T430 T443 T490 T516 T563 S581 S658 S784 S793 T950 S995 S1014 S1090 S1233 T85 S180 S258 S379 S412 T419 S575 T578 S742 T768 T807 T860 S903 Y366 Y733 Y53	N209 N277 N291 N328 N441 N606 N671 N830 N857		SHYC [Mus musculus] g3293551	BLAST-GenBank MOTIFS
9	98	S12 S44 T65	N91		Ariadne-2 protein (ARI2) [Homo sapiens] g3925604	BLAST-GenBank MOTIFS
10	524	T485 S29 T55 S63 S147 T177 S179 S209 S250 S254 T296 S471 S73 S193 S232 T237 S426	N125 N235 N336	Eyes absent developmental protein domain: P61-L524	Eya3 homolog [Mus musculus] g1816533	BLAST-GenBank MOTIFS BLAST-PRODOM
11	628	T466 T25 S56 S101 T182 T426 S501 T505 T606 T616 T209 S292 T310 T311 S459 S462 T498 S532 T590 Y251 Y562	N23 N142 N175	Signal peptide: M1-G46	ash 212 [Homo sapiens] g3046995	BLAST-GenBank MOTIFS SPSCAN

Table 2 (Cont.)

Polypeptide SEQ ID NO:	Amino Acid Residues	Potential Phosphorylation Sites	Potential Glycosylation Sites	Signature Sequence	Homologous Sequence	Analytical Methods and Databases
12	259	T202 S162 S175	N47 N94	Signal peptide: M1-M33 Transmembrane domain: Y204-V222 Leucine-rich repeat signature: L87-I100; L115-V128	SLIT1 protein [Homo sapiens] g4377995	BLAST-GenBank MOTIFS SPSCAN HMMER BLIMPS-PRINTS
13	380	S38 S43 T73 T194 T219 T297 Y329 Y377		Phosphatidyl-ethanolamine binding protein signature: L173-E203; G248-Q275 Lipid-binding protein domain: P169-L334	O-crystallin [Octopus dofleini] g4768844 Phosphatidyl-ethanolamine binding protein [Homo sapiens] g406290	BLAST-GenBank MOTIFS BLIMPS-BLOCKS BLAST-PRODOM BLAST-DOMO
14	130	T126 T104 T121	N111	Small proline-rich proteins motif: S2-P14; K27-C36 Feather keratin signature: M1-P39	skin-specific protein [Homo sapiens] g2589188	BLAST-GenBank MOTIFS BLIMPS-PRINTS BLAST-DOMO
15	761	S84 T123 T193 S457 T671 S2 S250 T269 T355 S382 S473 T485 S499 T557 S582 S622 T631 Y67 Y662	N148 N169	Signal peptide: M1-A60 ATP/GTP binding site motif A (P-loop): G578-T585	schlafen3 [Mus musculus] g3983152	BLAST-GenBank MOTIFS SPSCAN

Table 2 (Cont.)

Polypeptide SEQ ID NO:	Amino Acid Residues	Potential Phosphorylation Sites	Potential Glycosylation Sites	Signature Sequence	Homologous Sequence	Analytical Methods and Databases
16	197	T15 T109 T176 S47 S94 S142 S191 Y177	N104	Beta/gamma crystallin domains: S13-V98; R107-V195 Crystallins beta and gamma signature sequence: S2-E51; G41-N90 S89-G148; P131-T186	beta-A2 crystallin [Bos taurus] g162727	BLAST-GenBank MOTIFS HMMER-PFAM BLIMPS-BLOCKS PROFILESCAN BLAST-PRODOM BLAST-DOMO
17	339	T35 T160 S171 S207 S248 S309 T320 S25 S184 T224 Y281	N177 N332	Transmembrane domain: F103-I126	development-related protein [Rattus norvegicus] g4105412 Yamauchi, Y. et al. (1999) Brain Res. 68:149-158.	BLAST-GenBank MOTIFS HMMER
18	103			Octamer-binding transcription factor signature: Q6-Y21	small zinc finger-like protein [Homo sapiens] g5107198, g5107200	BLAST-GenBank MOTIFS BLIMPS-PRINTS
19	131	S7 T45 T71 T90 T2 S95 Y123		S7-P19: High mobility group protein K9-P19: AT hook-like domain signature I39-K130: Spindlin homolog Q49-K130: Testis specific protein	g3319677 Spindlin Homolog Oh, B. et al. (1997) Development 124:493-503	Motifs BLAST_GENBANK BLIMPS_PRINTS BLAST_PRODOM BLAST_DOMO
20	194	S31 T66	N59	D83-L92: Ribosomal protein L29 A153-L167: Vasopressin V2 receptor	g2245108 EREBP-4 (Ethylene-inducible DNA Binding Protein) -like protein	Motifs BLAST_GENBANK BLIMPS_BLOCKS BLIMPS_PRINTS

Table 2 (Cont.)

Polypeptide SEQ ID NO:	Amino Acid Residues	Potential Phosphorylation Sites	Potential Glycosylation Sites	Signature Sequence	Homologous Sequence	Analytical Methods and Databases
21	184	S35 S93 S16 T123		W106-T123: Brachyury protein family P25-Y42: Prostanoid EP2 receptor	g2078535 Nuclear protein E3-3 orf1	Motifs BLAST_GENBANK BLIMPS_PRINTS
22	528	T277 S3 T67 S99 S260 T291 S348 T452 T504 T173 T307	N430	L214-P226: Bromodomain protein motif V117-A135: Brain natriuretic peptidase motif A384-P394: Protein repeat neurofilament motif	g3860189 Wolf-Hirschhorn syndrome candidate 2 protein homolog Wright, T.J. et al. (1999) Genomics 59:203-212	Motifs BLAST_GENBANK BLIMPS_BLOCKS BLIMPS_PRINTS BLIMPS_PRODOM
23	298	T3 S122 S159 S172 S183 T235 T261 S277 T287 T114 S138 T265 Y149	N120 N273	P281-A291: Brain natriuretic peptidase motif	g1003016 Jerky gene product Toth,M. et al. (1995) Nat Genet 11:71-705	Motifs BLAST_GENBANK BLIMPS_PRINTS
24	630	T84 S102 S140 S17 T59 S111 S125 S165 S219 S314 S350 T376 S382 T416 S487 S491 S498 S522 S29 S32 T79 S95 S136 S141 S156 S201 S214 T244 S255 T310 T511 T595 S620 Y604	N404	D400-A415: Proenkephalin A precursor A33-C42: Gallidermin signature		Motifs BLIMPS_PRINTS

Table 2 (Cont.)

Polypeptide SEQ ID NO:	Amino Acid Residues	Potential Phosphorylation Sites	Potential Glycosylation Sites	Signature Sequence	Homologous Sequence	Analytical Methods and Databases
25	339	S27 S95 T185 T200 T222 T260 S286		P55-E70 Maspin signature	g1177322 CPG2 protein Nedivi,E. et al. (1996) Proc Natl Acad Sci U S A 93:2048-53	Motifs BLAST_GENBANK BLIMPS_PRINTS
26	189	S22 T169 T47 S116 S119 T160 Y31		D91-W101: Trp-Asp WD repeat D164-P173: Growth factor cystine knot D164: RGD domain	g4886904 Heme-binding protein	Motifs BLAST_GENBANK BLIMPS_BLOCKS BLIMPS_PRINTS
27	530	T88 T105 S119 T202 T215 T330 S427 S83 T92 T151 T354 S391 S467 S472	N450	M1-L19: Signal peptide M1-L18: Transmembrane motif L3-L16: Death domain	g2642446 Similar to auxin-responsive GH3 protein Abel, S. et al. (1995) J Biol Chem 270:19093-19099	Motifs BLAST_GENBANK HMMER SPSCAN BLIMPS_BLOCKS
28	356	S105 S202 S233 T253 T288 S312 T316 S338 S342 T350 T161 Y80 Y146 Y207	N159	M1-A26: Signal Peptide A136-Q153: Neuropeptide Y2 receptor	g4128223 Failed axon connections protein Geiger, E. et al. (1995) Genetics 141:595-606	Motifs SPSCAN BLAST_GENBANK BLIMPS_PRINTS

Table 3

Nucleotide SEQ ID NO:	Fragments	Tissue Expression (Fraction of Total)	Disease or Condition (Fraction of Total)	Vector
29	596-640	Nervous (0.800) Gastrointestinal (0.133) Endocrine (0.067)	Cell Proliferation (0.333) Inflammation/Trauma (0.267) Cancer (0.200)	pINCY
30	410-454	Developmental (0.421) Hematopoietic/Immune (0.211) Cardiovascular (0.158)	Cell Proliferation (0.579) Inflammation/Trauma (0.211) Cancer (0.105)	pINCY
31	147-191	Reproductive (0.300) Cardiovascular (0.160) Gastrointestinal (0.100) Musculoskeletal (0.100)	Cancer (0.480) Inflammation/Trauma (0.360) Cell Proliferation (0.160)	PSPORT1
32	463-507 658-702	Nervous (0.329) Reproductive (0.231) Gastrointestinal (0.091)	Cancer (0.490) Inflammation/Trauma (0.273) Cell Proliferation (0.175)	pINCY
33	111-155	Reproductive (1.000)	Inflammation/Trauma (1.000)	PBLUESCRIPT
34	812-856	Reproductive (0.500) Hematopoietic/Immune (0.250) Nervous (0.250)	Cancer (0.250) Inflammation/Trauma (0.250)	PBLUESCRIPT
35	350-394	Dermatologic (0.667) Urologic (0.333)	Cell Proliferation (0.667) Cancer (0.333)	PSPORT1
36	2254-2298 3739-3783	Nervous (0.306) Hematopoietic/Immune (0.194) Reproductive (0.112)	Cancer (0.357) Inflammation/Trauma (0.357) Cell Proliferation (0.184)	pINCY
37	386-430	Gastrointestinal (0.211) Cardiovascular (0.158) Reproductive (0.158)	Cancer (0.526) Inflammation/Trauma (0.369) Cell Proliferation (0.211)	PSPORT
38	137-181 746-790	Hematopoietic/Immune (0.333) Gastrointestinal (0.222) Nervous (0.222)	Inflammation/Trauma (0.667) Cancer (0.333) Cell Proliferation (0.222)	pINCY
39	183-233 2055-2099	Reproductive (0.269) Nervous (0.141) Hematopoietic/Immune (0.128)	Cancer (0.500) Cell Proliferation (0.269) Inflammation/Trauma (0.218)	pINCY
40	704-748	Nervous (0.600) Developmental (0.200) Reproductive (0.200)	Cancer (0.400) Cell Proliferation (0.200)	pINCY
41	164-208 1115-1159	Reproductive (0.250) Nervous (0.164) Cardiovascular (0.129)	Cancer (0.457) Inflammation/Trauma (0.310) Cell Proliferation (0.198)	pINCY

Table 3 (Cont.)

Nucleotide SEQ ID NO:	Fragments	Tissue Expression (Fraction of Total)	Disease or Condition (Fraction of Total)	Vector
42	399-443	Cardiovascular (0.333) Developmental (0.333) Reproductive (0.333)	Cancer (0.333) Cell Proliferation (0.333)	pINCY
43	507-551 1539-1583	Reproductive (0.385) Developmental (0.308) Gastrointestinal (0.154)	Cancer (0.462) Cell Proliferation (0.462)	pINCY
44	551-595	Endocrine (0.333) Gastrointestinal (0.333) Reproductive (0.333)	Cancer (1.000)	pINCY
45	1037-1081 1145-1189	Nervous (0.566) Cardiovascular (0.132)	Inflammation/Trauma (0.408) Cancer (0.184) Cell Proliferation (0.105)	pINCY
46	22-66	Reproductive (0.268) Hematopoietic/Immune (0.196) Nervous (0.143)	Cancer (0.446) Inflammation/Trauma (0.340) Cell Proliferation (0.214)	pINCY
47	1-375 694-861	Nervous (0.400) Reproductive (0.240) Gastrointestinal (0.160)	Cancer (0.520) Inflammation/Trauma (0.280) Cell proliferation (0.120)	PSPORT1
48	1-73 572-3860	Reproductive (0.242) Cardiovascular (0.147) Hematopoietic/Immune (0.137)	Cancer (0.495) Inflammation/Trauma (0.337) Cell proliferation (0.179)	PSPORT1
49	1-188 605-726	Reproductive (0.265) Cardiovascular (0.147) Nervous (0.140)	Cancer (0.485) Cell proliferation (0.176) Inflammation/Trauma (0.287)	pINCY
50	1-85 470-754 809-889 995-1030 1442-2196	Reproductive (0.308) Nervous (0.250) Hematopoietic/Immune (0.115)	Cancer (0.538) Inflammation/Trauma (0.288) Cell proliferation (0.154)	pINCY
51	1-540 601-1059 1438-1495	Nervous (0.600) Cardiovascular (0.200) Gastrointestinal (0.200)	Cancer (0.600) Inflammation/Trauma (0.400) Neurological (0.200)	pINCY
52	1-283 971-1069 1682-2794	Nervous (0.615) Gastrointestinal (0.103) Reproductive (0.103)	Cancer (0.513) Inflammation/Trauma (0.359) Trauma (0.154)	PSPORT1

Table 3 (Cont.)

Nucleotide SEQ ID NO:	Fragments	Tissue Expression (Fraction of Total)	Disease or Condition (Fraction of Total)	Vector
53	1-1516	Nervous (0.333) Reproductive (0.333) Developmental (0.167) Musculoskeletal (0.167)	Cancer (0.667) Cell proliferation (0.167) Inflammation/Trauma (0.167)	PSPORT1
54	1-133 704-1146	Reproductive (0.384) Nervous (0.178) Cardiovascular (0.110)	Cancer (0.479) Cell proliferation (0.164) Inflammation/Trauma (0.274)	PSPORT1
55	1-1050 1204-1320 1621-2761	Nervous (0.208) Reproductive (0.208) Developmental (0.146)	Cancer (0.354) Inflammation/Trauma (0.375) Cell proliferation (0.188)	PSPORT1
56	1-899 1075-1164	Nervous (0.444) Urologic (0.222) Reproductive (0.111) Gastrointestinal (0.111) Developmental (0.111)	Cancer (0.444) Cell proliferation (0.222) Inflammation/Trauma (0.222)	pINCY

Table 4

Nucleotide SEQ ID NO:	Library	Library Description
29	STOMFET01	This library was constructed using RNA isolated from the stomach tissue of a Caucasian female fetus, who died at 20 weeks' gestation.
30	UCMCNOT02	This library was constructed using RNA isolated from mononuclear cells obtained from the umbilical cord blood of nine individuals.
31	LUNGTUT03	This library was constructed using RNA isolated from lung tumor tissue removed from the left lower lobe of a 69-year-old Caucasian male during segmental lung resection. Pathology indicated residual grade 3 invasive squamous cell carcinoma. Patient history included acute myocardial infarction, prostatic hyperplasia, malignant skin neoplasm, and tobacco use.
32	CORPNOT02	This library was constructed using RNA isolated from diseased corpus callosum tissue removed from the brain of a 74-year-old Caucasian male who died from Alzheimer's disease.
33	TESTNOT03	This library was constructed using RNA isolated from testicular tissue removed from a 37-year-old Caucasian male, who died from liver disease. Patient history included cirrhosis, jaundice, and liver failure.
34	TESTNOT03	This library was constructed using RNA isolated from testicular tissue removed from a 37-year-old Caucasian male, who died from liver disease. Patient history included cirrhosis, jaundice, and liver failure.
35	KERANOT02	This library was constructed using RNA isolated from epidermal breast keratinocytes (NHEK). NHEK (Clontech #CC-2501) is a human breast keratinocyte cell line derived from a 30-year-old black female during breast-reduction surgery.
36	LUNGTUT10	This library was constructed using RNA isolated from lung tumor tissue removed from the left upper lobe of a 65-year-old Caucasian female during a segmental lung resection. Pathology indicated a metastatic grade 2 myxoid liposarcoma and a metastatic grade 4 liposarcoma. Patient history included soft tissue cancer, breast cancer, and secondary lung cancer.

Table 4 (Cont.)

Nucleotide SEQ ID NO:	Library	Library Description
37	THP1AZS08	This subtracted THP-1 promonocyte cell line library was constructed using 5.76 million clones from a 5-aza-2'-deoxycytidine (AZ) treated THP-1 cell library. Starting RNA was made from THP-1 promonocyte cells treated for three days with 0.8 micromolar AZ. The hybridization probe for subtraction was derived from a similarly constructed library, made from RNA isolated from untreated THP-1 cells. 5.76 million clones from the AZ-treated THP-1 cell library were then subjected to two rounds of subtractive hybridization with 5 million clones from the untreated THP-1 cell library. Subtractive hybridization conditions were based on the methodologies of Swaroop et al., NAR (1991) 19:1954, and Bonaldo et al., Genome Research (1996) 6:791. THP-1 (ATCC TIB 202) is a human promonocyte line derived from peripheral blood of a 1-year-old Caucasian male with acute monocytic leukemia (Int. J. Cancer 26 (1980):171).
38	BRSTNOT12	This library was constructed using RNA isolated from diseased breast tissue removed from a 32-year-old Caucasian female during a bilateral reduction mammoplasty. Pathology indicated nonproliferative fibrocystic disease. Family history included benign hypertension and atherosclerotic coronary artery disease.
39	BRSTTUT13	This library was constructed using RNA isolated from breast tumor tissue removed from the right breast of a 46-year-old Caucasian female during a unilateral extended simple mastectomy with breast reconstruction. Pathology indicated an invasive grade 3 adenocarcinoma, ductal type with apocrine features and greater than 50% intraductal component. Patient history included breast cancer.
40	KIDNFET01	This library was constructed using RNA isolated from kidney tissue removed from a Caucasian female fetus, who died at 17 weeks' gestation from anencephalus.
41	BRAIFET01	This library was constructed using RNA isolated from brain tissue removed from a Caucasian male fetus, who was stillborn with a hypoplastic left heart at 23 weeks' gestation.
42	SKINNOT04	This library was constructed using RNA isolated from breast skin tissue removed from a 70-year-old Caucasian female during a breast biopsy and resection.
43	OVARNOT11	This library was constructed using RNA isolated from right ovarian tissue removed from a 43-year-old Caucasian female during a total abdominal hysterectomy and bilateral salpingooophorectomy with dilation and curettage. The posterior serosa contained a focus of endometriosis. Pathology for the associated tumor tissue indicated multiple (1 submucosal, 4 intramural) leiomyomata. Family history included atherosclerotic coronary artery disease, lung cancer, benign hypertension, and a kidney transplant.

Table 4 (Cont.)

Nucleotide SEQ ID NO:	Library	Library Description
44	ADRETUT07	This library was constructed using RNA isolated from adrenal tumor tissue removed from a 43-year-old Caucasian female during a unilateral adrenalectomy. Pathology indicated pheochromocytoma.
45	SYNONOT01	This library was constructed using RNA isolated from synovial tissue removed from a 75-year-old Caucasian male.
46	LUNGNOT31	This library was constructed using RNA isolated from right middle lobe lung tissue removed from a 63-year-old Caucasian male. Pathology for the associated tumor indicated grade 3 adenocarcinoma. Patient history included an abdominal aortic aneurysm, cardiac dysrhythmia, atherosclerotic coronary artery disease, hiatal hernia, chronic sinusitis, and lupus. Family history included acute myocardial infarction and atherosclerotic coronary artery disease.
47	BRSTNOT03	This library was constructed using RNA isolated from diseased breast tissue from a 54-year-old Caucasian female. Family history included benign hypertension, hyperlipidemia and a malignant neoplasm of the colon.
48	BRSTTUT03	This library was constructed using RNA isolated from breast tumor tissue from a 58-year-old Caucasian female. Patient history included skin cancer, rheumatic heart disease, osteoarthritis, and tuberculosis. Family history included cerebrovascular disease, coronary artery aneurysm, breast cancer, prostate cancer, atherosclerotic coronary artery disease, and type I diabetes.
49	LUNGNOT12	This library was constructed using RNA isolated from lung tissue from a 78-year-old Caucasian male. Patient history included cerebrovascular disease, arteriosclerotic coronary artery disease, thrombophlebitis, chronic obstructive pulmonary disease, and asthma. Family history included intracranial hematoma, cerebrovascular disease, arteriosclerotic coronary artery disease, and type I diabetes.
50	BRAITUT08	This library was constructed using RNA isolated from brain tumor tissue from the left frontal lobe of a 47-year-old Caucasian male. Pathology indicated astrocytoma with focal tumoral radionecrosis. Patient history included cerebrovascular disease, deficiency anemia, hyperlipidemia, epilepsy, and tobacco use. Family history included cerebrovascular disease and a malignant prostate neoplasm.
51	PANCNOT08	This library was constructed using RNA isolated from pancreatic tissue from a 65-year-old Caucasian female. Patient history included type II diabetes, osteoarthritis, cardiovascular disease, benign neoplasm in the large bowel, and a cataract. Family history included cardiovascular disease, type II diabetes, and stomach cancer.

Table 4 (Cont.)

Nucleotide SEQ ID NO:	Library	Library Description
52	PROSTUT04	This library was constructed using RNA isolated from prostate tumor tissue from a 57-year-old Caucasian male. Patient history included a benign neoplasm of the large bowel and type I diabetes. Family history included a malignant neoplasm of the prostate and type I diabetes.
53	PROSTUT05	This library was constructed using RNA isolated from prostate tumor tissue from a 69-year-old Caucasian male. Adenofibromatous hyperplasia was also present. Family history included congestive heart failure, multiple myeloma, hyperlipidemia, and rheumatoid arthritis.
54	OVARNOT03	This library was constructed using RNA isolated from ovarian tissue removed from a 43-year-old Caucasian female. Pathology for the associated tumor tissue indicated mucinous cystadenocarcinoma. Patient history included mitral valve disorder, pneumonia, and viral hepatitis. Family history included atherosclerotic coronary artery disease, pancreatic cancer, stress reaction, cerebrovascular disease, breast cancer, and uterine cancer.
55	HIPOAZT01	This library was constructed from RNA isolated from diseased hippocampus tissue from the brain of a 74-year-old Caucasian male who died from Alzheimer's disease.
56	ESOGNOT03	This library was constructed using RNA isolated from esophageal tissue from a 53-year-old Caucasian male. Patient history included membranous nephritis, hyperlipidemia, benign hypertension, and anxiety state. Family history included atherosclerotic coronary artery disease, cirrhosis, abdominal aortic aneurysm rupture, breast cancer, myocardial infarction, and atherosclerotic coronary artery disease.

Table 5

Program	Description	Reference	Parameter Threshold
ABI FACTURA	A program that removes vector sequences and masks ambiguous bases in nucleic acid sequences.	Perkin-Elmer Applied Biosystems, Foster City, CA.	
ABI/PARACEL FDF	A Fast Data Finder useful in comparing and annotating amino acid or nucleic acid sequences.	Perkin-Elmer Applied Biosystems, Foster City, CA; Paracel Inc., Pasadena, CA.	Mismatch <50%
ABI AutoAssembler	A program that assembles nucleic acid sequences.	Perkin-Elmer Applied Biosystems, Foster City, CA.	
BLAST	A Basic Local Alignment Search Tool useful in sequence similarity search for amino acid and nucleic acid sequences. BLAST includes five functions: blastp, blastn, blastx, tblastn, and tblastx.	Altschul, S.F. et al. (1990) J. Mol. Biol. 215:403-410; Altschul, S.F. et al. (1997) Nucleic Acids Res. 25: 3389-3402.	*ESTs:* Probability value= 1.0E-8 or less *Full Length sequences*: Probability value= 1.0E-10 or less
FASTA	A Pearson and Lipman algorithm that searches for similarity between a query sequence and a group of sequences of the same type. FASTA comprises as least five functions: fasta, tfasta, fastx, tfastx, and ssearch.	Pearson, W.R. and D.J. Lipman (1988) Proc. Natl. Acad Sci. 85:2444-2448; Pearson, W.R. (1990) Methods Enzymol. 183: 63-98; and Smith, T.F. and M. S. Waterman (1981) Adv. Appl. Math. 2:482-489.	*ESTs:* fasta E value=1.06E-6 *Assembled ESTs*: fasta Identity= 95% or greater and Match length=200 bases or greater; fastx E value=1.0E-8 or less *Full Length sequences:* fastx score=100 or greater
BLIMPS	A BLocks IMProved Searcher that matches a sequence against those in BLOCKS, PRINTS, DOMO, PRODOM, and PFAM databases to search for gene families, sequence homology, and structural fingerprint regions.	Henikoff, S and J.G. Henikoff, Nucl. Acid Res., 19:6565-72, 1991. J.G. Henikoff and S. Henikoff (1996) Methods Enzymol. 266:88-105; and Attwood, T.K. et al. (1997) J. Chem. Inf. Comput. Sci. 37: 417-424.	Score=1000 or greater; Ratio of Score/Strength = 0.75 or larger; and, if applicable, Probability value= 1.0E-3 or less
HMMER	An algorithm for searching a query sequence against hidden Markov model (HMM)-based databases of protein family consensus sequences, such as PFAM.	Krogh, A. et al. (1994) J. Mol. Biol., 235:1501-1531; Sonnhammer, E.L.L. et al. (1988) Nucleic Acids Res. 26:320-322.	Score=10-50 bits for PFAM hits, depending on individual protein families

Table 5 (cont.)

Program	Description	Reference	Parameter Threshold
ProfileScan	An algorithm that searches for structural and sequence motifs in protein sequences that match sequence patterns defined in Prosite.	Gribskov, M. et al. (1988) CABIOS 4:61-66; Gribskov, et al. (1989) Methods Enzymol. 183:146-159; Bairoch, A. et al. (1997) Nucleic Acids Res. 25: 217-221.	Normalized quality score≥GCG-specified "HIGH" value for that particular Prosite motif. Generally, score=1.4-2.1.
Phred	A base-calling algorithm that examines automated sequencer traces with high sensitivity and probability.	Ewing, B. et al. (1998) Genome Res. 8:175-185; Ewing, B. and P. Green (1998) Genome Res. 8:186-194.	
Phrap	A Phils Revised Assembly Program including SWAT and CrossMatch, programs based on efficient implementation of the Smith-Waterman algorithm, useful in searching sequence homology and assembling DNA sequences.	Smith, T.F. and M. S. Waterman (1981) Adv. Appl. Math. 2:482-489; Smith, T.F. and M. S. Waterman (1981) J. Mol. Biol. 147:195-197; and Green, P., University of Washington, Seattle, WA.	Score= 120 or greater; Match length= 56 or greater
Consed	A graphical tool for viewing and editing Phrap assemblies	Gordon, D. et al. (1998) Genome Res. 8:195-202.	
SPScan	A weight matrix analysis program that scans protein sequences for the presence of secretory signal peptides.	Nielson, H. et al. (1997) Protein Engineering 10:1-6; Claverie, J.M. and S. Audic (1997) CABIOS 12: 431-439.	Score=3.5 or greater
Motifs	A program that searches amino acid sequences for patterns that matched those defined in Prosite.	Bairoch et al. supra; Wisconsin Package Program Manual, version 9, page M51-59, Genetics Computer Group, Madison, WI.	